Synaptic tagging and capture mechanisms during the formation of memory: An exploratory study

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Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated, and that this work has not been submitted for any other degree or professional qualification. Experiments 1, 6 and 7 were conducted by Dr. Tobias Bast. Autoradiographic imaging and quantitative densitometric analysis in Expt. 19 were conducted by Dr. Paul Kelly and Dr. Harry Olverman. In situ hybridization and immunohistochemistry protocols in Expt. 21 were conducted by Dr. Almira Vazdarjanova.

(*Bruno Teixeira da Silva*)

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In memory of
my mother Maria Teresa and my aunt Maria Margarida

Abstract

In everybody's lives, there are strong emotional or surprising events that, for being special, are vividly remembered for a lifetime. Sometimes, these memories include one-shot images or details of associated daily life events that, for being ordinary, should have been rapidly forgotten. Why and how does the brain form and retain detailed memories of trivial events? The synaptic tagging and capture (STC) hypothesis of memory formation (Frey & Morris, Nature 1997) provides a theoretical framework that might explain the formation of these *flashbulb memories* at a cellular level. The hypothesis suggests that *strong* events, producing long-lasting memories, might stabilise memory for *weak* events by up-regulating the synthesis of late-phase plasticity-related proteins in neurons encoding memory traces for both events. This thesis tests this prediction of the STC hypothesis during the formation of long-term place memory in rodents.

First, two new behavioural tasks are developed which provide sensitive measures of rapidly acquired place memory persistence - a new one-trial place memory task in the "event arena" and a modified delayed matching-to-place (DMP) protocol in the watermaze. Persistence of place memory is assessed and compared in these tasks. Given the important role of NMDA receptor activation during STC mechanisms, the contribution of NMDA and AMPA receptor activation in the hippocampus for the encoding and retrieval of place memory, respectively, is also established. Finally, *weak* and *strong* encoding events, leading to the formation of either short- or long-lasting place memory in the watermaze DMP task, are characterized.

A second series of experiments investigates the possibility of synergistic interactions between different encoding events that occur in two different watermazes. First, weak and strong encoding events are arranged to occur within a short time-window to test behavioural analogues of the "strong-before-weak" and "weak-before-strong" STC paradigms characterised in electrophysiological experiments in rat hippocampal slices (Frey and Morris, 1997, 1998b). Then, after establishing *i)* the time course and local specificity of protein synthesis inhibition by intra-hippocampal infusion of anisomycin *in vivo*, *ii)* the dependence of long-term memory for strong encoding events on protein synthesis in the hippocampus, and *iii)* the induction of transcriptional and translational mechanisms in the hippocampus by strong encoding events, a behavioural analogue of the "strong-before-strong" STC paradigm (Frey and Morris, 1997) is also investigated.

The results of these experiments are supportive of *i)* a role for hippocampal NMDA receptor-mediated synaptic plasticity in the encoding of rapidly acquired place memory; *ii)* a role for hippocampal AMPA receptor-mediated synaptic transmission in both encoding and retrieval of memory; and *iii)* a role for transcriptional and translational mechanisms in the hippocampus in the stabilisation of place memory. However, no evidence could be found supporting the involvement of synaptic tagging and capture mechanisms during the formation of long-lasting place memory.

Table of Contents

List of Figures

Supplemental data

Chapter 1: Introduction

1.1. Synaptic plasticity and memory formation

One of the most captivating and influential ideas in neuroscience is that organisms learn by activity-dependent changes of communication between nerve cells. In mammals, this idea was strongly supported by two major discoveries in the second half of the twentieth century. The first was the essential role of the human hippocampus and related medial temporal lobe structures in the formation of new long-lasting memories (Scoville and Milner, 1957), the second was the detection of altered synaptic transmission efficiency *in vivo* following electrical stimulation of a major pathway of the rodent hippocampal circuitry (Bliss and Lomo, 1973). Together, these two key findings set the grounds for the now widely investigated idea that the formation of some types of memory in humans and non-human mammals is mediated by mechanisms of activity-dependent synaptic plasticity in the hippocampus (Martin et al., 2000b).

The memory

The essential work of William Scoville and Brenda Milner with amnesic patients, as well as that of others, revealed two main consequences of bilateral damage to the hippocampus. *First*, the loss of recently formed memories, *second*, the loss of the ability to form new long-lasting memories (reviewed by Spiers et al., 2001). These observations were crucial not only because they pointed to the hippocampus as a critical brain structure in the formation of memory, but also because they suggested that memory formation relied on gradual systems-level consolidation mechanisms, only partially mediated by the hippocampus (reviewed by Squire and Alvarez, 1995).

Since these original studies, the human hippocampus has been widely implicated in the formation of several types of memory that have been addressed collectively as declarative/explicit memories (knowing what), as opposed to non-declarative/implicit memories (knowing how), which are spared in amnesia (Squire, 1992; Squire and Zola, 1996; Eichenbaum, 1997a, 2001). Human declarative memories consist of memories that can be declared or brought to mind as a proposition or an image, including autobiographical episodes (episodic memory) and facts (semantic memory) (Tulving, 1984; Squire, 1992; Tulving, 2002; Manns et al., 2003a; Squire et al., 2004). Controversy remains as to whether the human hippocampus plays a selective role in episodic memory or a more general role in both episodic and semantic memories (Vargha-Khadem et al., 1997; Squire and Zola, 1998; Tulving and Markowitsch, 1998; Manns et al., 2003a). Also falling within the broad definition of declarative memories, both the capacity to correctly remember something that has been encountered before, i.e. recognition memory (Reed and Squire, 1997; Brown and Xiang, 1998; Brown and Aggleton, 2001; Manns et al., 2003b; Eichenbaum et al., 2007; Squire et al., 2007) and the capacity to form cognitive maps and use them during navigation through space, i.e. spatial memory (Bohbot et al., 1998; Kessels et al., 2001; Burgess et al., 2002; Feigenbaum and Morris, 2004; Parslow et al., 2004; Parslow et al., 2005), have been equally associated to the human hippocampus.

Performance in a series of different behavioural tasks for rodents has been also shown to depend on hippocampal integrity. Common features of these tasks include relational memories (memories for relations among stimuli and events) and memories that utilize spatial-contextual information, both of which seem reasonable analogues of human declarative memory (Eichenbaum et al., 1996; Thompson and Kim, 1996; Eichenbaum, 2001). Given that both the human and rodent hippocampus have been implicated in the formation of so many different sub-types of memory it is maybe not surprising that determining a single specific hippocampal function has not been an easy task. Indeed, the precise functions of the hippocampus and its various sub-regions are still under intense debate. Integrative neuropsychological theories of hippocampal function have not only suggested that the hippocampus plays a specific role in cognitive mapping (O'Keefe and Nadel, 1978; Burgess et al., 2002), declarative memory (Squire, 1992), episodic memory and certain aspects of episodic memory (Tulving, 1984; Morris and Frey, 1997; Vargha-Khadem et al., 1997; Aggleton and Brown, 1999; Morris, 2006), but there have also been longstanding and newer ideas about context specific encoding and retrieval of specific events (Hirsh, 1974), and the rapid acquisition of configural or conjunctive associations (O'Reilly and Rudy, 2001). This thesis takes a neutral stand on these debates, but uses place memory tasks to investigate a specific aspect of the synaptic plasticity and memory hypothesis (see below). It is not of consequence that place memory is or is not a subset of declarative memory, to consider just one of these neuropsychological debates.

The structure

In addition to the hippocampal formation, which comprises the *Cornu Ammonis* (CA1 and CA3 regions), the dentate gyrus (DG) and the subiculum, other medial temporal structures that mediate memory processes, including memory consolidation in rodents, are the perirhinal, postrhinal and entorhinal cortices, which collectively comprise the parahippocampal region (Burwell et al., 1995; Eichenbaum et al., 1996; Eichenbaum, 2000).

Multi-sensory information directed to the hippocampus has its origin practically in all neocortical association areas (Burwell and Amaral, 1998). Each of these neocortical areas projects to one or more of the interconnected parahippocampal regions and send main efferents to the hippocampal formation, providing a site for cortical input convergence. The hippocampal formation receives information from the neocortex and the cingulate cortex via the entorhinal cortex and from subcortical areas via the fornix; it then sends its outputs via the subiculum and the fornix back to the same cortical and subcortical structures. Processed hippocampal outcome is therefore returned to the parahippocampal regions, which in turn, relay back to neocortical areas (Burwell et al., 1995).

Some lines of evidence suggest that at least some types of hippocampal-dependent memory are stored in the same associative neocortical structures involved in processing of relevant information during learning (e.g. Nyberg et al., 2000). According to the "standard" systems-level model of memory consolidation, in an early stage the hippocampus is thought to connect the neocortical regions and to allow memory to be reactivated for retrieval. With time, the connections among the neocortical regions are progressively strengthened until the cortical memory can be reactivated and retrieved independently of the hippocampus (Squire and Alvarez, 1995; Manns et al., 2003b; Squire et al., 2004). Controversy as to the general applicability of this model to all types of hippocampal-dependent memory was raised with the consistent observation of flat graded retrograde amnesia for spatial memory in several studies (reviewed by Morris, 2006). These studies suggest that the integrity of the hippocampus may always be required for the recall of spatial memory.

Transverse sections along the septo-temporal axis of the hippocampus reveal a lamellar organization with a preserved tri-synaptic circuitry. Long considered one of the fundamental information processing pathways in the hippocampal formation, able to mediate a large network of associations, the tri-synaptic circuitry has its major input in the perforant pathway, which originates from stellate excitatory neurons of layer II of the entorhinal cortex and establishes

synapses with granule cells of the DG (Amaral and Lavenex, 2007). Axons from DG granular cells, called mossy fibers, project to CA3 region and establish synapses with the apical dendrites of its pyramidal cells. Projections from these cells form the Schaffer collateral pathway terminating on the apical dendrites of CA1 pyramidal cells in the *stratum radiatum*. Axons from these cells constitute the major hippocampal output pathway projecting back to the subiculum and EC as well as subcortical targets (Amaral and Lavenex, 2007). Another input from the entorhinal cortex to the hippocampus, the direct or temporoammonic pathway, consists of axons from layer III entorhinal cortex neurons that establish synapses mainly on the distal dendrites of CA1 neurons in the *stratum lacunosum-moleculare* (Steward and Scoville, 1976; Witter et al., 1989). Although there is some controversy as to whether the direct pathway input is primarily excitatory or inhibitory (Soltesz and Jones, 1995), described projections of the tri-synaptic circuitry are all known to be glutamatergic and excitatory (Misgeld, 1988), having each hippocampal sub-region local GABAergic interneurons that provide feedback and feed-forward inhibition (Woodson et al., 1989; Freund and Buzsaki, 1996).

In hippocampal excitatory synapses, an action potential results in release of glutamate stored in vesicles in axon terminals. Different classes of glutamate receptors in the postsynaptic membrane subsequently transduce the glutamate signal into electrical and biochemical events in the postsynaptic neuron (Nakanishi et al., 1998; Dingledine et al., 1999). The cationic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor channel, permeable to calcium and sodium ions, opens in response to glutamate binding and mediates most of the rapid excitatory postsynaptic current (reviewed by Dingledine et al., 1999). The NMDA (*N*-methyl-D-aspartate) receptor channel, with high permeability to calcium, opens in response to glutamate but only when the postsynaptic membrane is depolarized beyond -40mV, leading to the relief of the Mg^{2+} block (Mayer et al., 1984; Nowak et al., 1984; Herron et al., 1986). This has the effect of NMDA receptors acting as cellular coincidence detectors requiring two

simultaneous events to be activated: *first*, the binding of glutamate released from presynaptic terminals and *second*, sufficient postsynaptic depolarization mediated by AMPA receptors.

The mechanism

In 1973, Bliss and Lømo published a groundbreaking study showing that repeated high-frequency electrical stimulation of the perforant path in the hippocampus of anaesthetised rabbits induced a long-lasting (up to 10h) strengthening of the synaptic connections between these fibres and their target cells, the granular cells of the dentate gyrus. Strengthening was only confined to stimulated synapses and, therefore, input-specific (Bliss and Lomo, 1973; Andersen et al., 1977; Lynch et al., 1977). The discovery of this phenomenon, subsequently termed as homosynaptic long-term potentiation (LTP), was extremely important because, as referred above, it showed for the first time that activity-dependent changes in synaptic efficiency were possible in a brain structure implicated in the formation of memory (Scoville and Milner, 1957). The progressive characterization of the mechanisms and properties of homosynaptic LTP in the ensuing years further strengthened the idea that this type of physiological phenomenon could be a suitable candidate for the cellular basis of learning and memory. LTP was rapidly observed not only in the three main pathways in the rodent hippocampus but also in other brain regions and in other species (reviewed by Bliss and Lynch, 1988), and soon, induction of homosynaptic LTP in both CA3-CA1 and DG-CA3 rat hippocampal synapses was shown to depend on the activation of NMDA receptors (Collingridge et al., 1983; Harris et al., 1984; Coan et al., 1987; Errington et al., 1987). Given the above-described properties of NMDA receptor activation, the dependence of LTP induction on the activation of this type of receptors further suggested the involvement of homosynaptic "associative" induction mechanisms resembling Donald Hebb's postulate for associative learning (Hebb, 1949).

Hebb had proposed that learning reflected the selective strengthening of synapses in response to coincident activity of both presynaptic and postsynaptic neurons. In his own now famous words Hebb suggested that:

"When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficacy, as one of the cells firing B, is increased…" and " any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other" (Hebb, 1949).

Early electrophysiological experiments had shown that when subthreshold tetanic stimulation was applied simultaneously, or nearly simultaneously (within a range of milliseconds), to two independent pathways projecting to the same population of hippocampal neurons, LTP would often be produced in both pathways when neither input, on its own, was able to do so (McNaughton et al., 1978). Strong stimulation of one pathway would also lead to potentiation in a second weakly stimulated pathway (Levy and Steward, 1979; Barrionuevo and Brown, 1983; Gustafsson and Wigström, 1986), and the degree of potentiation obtained in a single input would depend on the strength of stimulation used for tetanic activation (McNaughton et al., 1978). Overall, these experiments suggested that both the probability and the magnitude of LTP depended on "cooperative" (McNaughton et al., 1978) or "associative" (Levy and Steward, 1979) mechanisms determined by the number of afferent fibres recruited homosynaptically or heterosynaptically. Associativity, which would be later associated specifically to heterosynaptic stimulation paradigms (Wigstrom et al., 1993), further implied that a certain synapse was only potentiated when its afferent was active in temporal association with sufficient activity in other afferents. Thus, input-specificity and associativity/cooperativity complied with Hebb's description of synaptic strengthening, and NMDA receptor activation provided a putative neurophysiological basis for these phenomena. Following the first evidence for a requirement for NMDA receptor activation during induction of LTP in the hippocampus

(Collingridge et al., 1983; Harris et al., 1984) were the first studies suggesting that Hebbian associative mechanisms could indeed be involved in the induction of LTP in that same brain region (Kelso et al., 1986; Gustafsson and Wigstrom, 1988; Kauer et al., 1988).

The above described "classical" properties of "Hebbian" LTP, i.e. input-specificity, associativity and persistence, are highly suggestive of an information storage mechanism not only because associative induction is an appealing candidate as a cellular substrate of associative learning, but also because input-specificity greatly improves the storage capacity of neural networks (Sejnowski, 1977; Dayan and Willshaw, 1991), and because persistent changes have to occur in the brain in order for it to retain information. Indeed, the first studies implicating LTP in memory have shown a close parallel between the persistence of hippocampal LTP and the persistence of hippocampal-dependent memories (Barnes, 1979; Barnes and McNaughton, 1985), and more recently described properties of LTP meet the expectations of a mechanism sustaining long-lasting memory (recently reviewed by Abraham and Williams, 2008). Critically, there is also some evidence that natural patterns of cell firing in behaving animals are effective in inducing LTP in the hippocampus (Dobrunz and Stevens, 1999).

Although other forms of neuronal excitability might underlie some types of learning and memory (recently reviewed by Kim and Linden, 2007; Nelson and Turrigiano, 2008; Mozzachiodi and Byrne, 2009), and some alternative accounts of LTP function have also been proposed (e.g. Shors and Matzel, 1997; Arshavsky, 2006), it is now widely accepted that there is a causal link between LTP, as well as other forms of activity-dependent synaptic plasticity [such as long-term depression (LTD; Dudek and Bear, 1992) and depotentiation (DP; Barrionuevo et al., 1980)], and learning and memory (reviewed by Bliss and Collingridge, 1993; Bear, 1999; Bliss et al., 2007; Hansel and Bear, 2009; Sweatt, 2009).

This general idea, which is usually addressed to as "synaptic plasticity and memory" (SPM) hypothesis, assumes that:

"Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed" (Martin et al., 2000b; Martin and Morris, 2002).

Martin and colleagues have suggested that in order for this hypothesis to be upheld there are a set of criteria that have to be met experimentally: *first*, the DETECTABILITY criteria states that if an animal displays memory for an experience, then, a change in synaptic efficacy should also be detectable in the nervous system; *second*, the RETROGRADE ALTERATION and the ANTEROGRADE ALTERATION criteria state that interventions that *i)* alter the spatial distribution of synaptic changes induced by a prior learning experience or *ii)* prevent the induction of synaptic weight changes during the learning experience, should also impair the animal's memory of that experience; *finally*, the MIMICRY criteria states if it were possible to induce the appropriate pattern of synaptic weight changes artificially, the animal should display "apparent" memory for some past experience which did not occur (Martin et al., 2000b; Martin and Morris, 2002).

Detectability, anterograde alteration and retrograde alteration, refer to the necessity of synaptic plasticity for information storage, mimicry refers to its sufficiency. Both pharmacological and genetic studies have now provided a wide diversity of compelling evidence supporting the notion of necessity (for recent reviews see Bliss et al., 2007; Neves et al., 2008). To test the notion of sufficiency, which implies memory without learning, it is still beyond the current stage of knowledge. As suggested somewhere else (Neves et al., 2008), a possible approach to this problem would be to create a memory by a standard training procedure, erase it, and subsequently re-create it by exploiting the knowledge obtained about the synaptic changes that took place during learning.

Most studies addressing the necessity criteria have investigated interventions that blocked the induction or expression of synaptic plasticity in the hippocampus and disrupted the formation of hippocampal-dependent memory, with no apparent non-specific effects. Worth mentioning is the first of these studies which showed that intraventricular infusion of the selective NMDA receptor antagonist AP5 blocked the induction of LTP in the hippocampus *in vivo* and profoundly impaired the formation of place memory in the watermaze task, while having no significant effect on basal transmission (Morris et al., 1986). This study pioneered a wide range of studies that have now strongly implicated NMDA-mediated hippocampal synaptic plasticity mechanisms in the formation of hippocampal-dependent spatial, and non-spatial, memories (Morris, 1989; Ward et al., 1990; Davis et al., 1992; Tsien et al., 1996; Steele and Morris, 1999; Nakazawa et al., 2002; Day et al., 2003; Nakazawa et al., 2003; Riedel et al., 2003; Nakazawa et al., 2004). A similar type of study has also recently shown that infusion of ZIP (myristoylated zeta-pseudosubstrate inhibitory peptide), a specific inhibitor of the protein kinase PKMζ (a persistently active isoform of protein kinase C) into the hippocampus, impairs the maintenance of LTP and spatial memory without producing detectable non-specific effects (Pastalkova et al., 2006). Comparable effects of PKMζ inhibition on other types of memory have also been documented (Shema et al., 2007; Serrano et al., 2008). Finally, it is important to mention that compelling evidence supporting the SPM hypothesis has been very recently provided by three studies suggesting that associative learning produces LTP-like changes in the rodent hippocampus (Gruart et al., 2006; Whitlock et al., 2006) and that sensory-dependent learning may be sustained by plasticity-like changes in the rodent neocortex (Clem et al., 2008).

Induction of NMDA-dependent synaptic plasticity phenomena results from the transient NMDA-mediated increase of intracellular calcium in postsynaptic cells. This leads to the activation of numerous synaptic proteins (e.g. protein kinases/phosphatases) via posttranslational modifications and protein-protein interactions that progressively lead to enduring modifications of the synapses (e.g. by changing the conductance and/or number of

AMPA receptors in the postsynaptic membrane). In order to persist, not only synaptic plasticity mechanisms, but also memory mechanisms, require protein synthesis. Both these mechanisms exhibit at least two distinct temporal phases: an *early-phase*, lasting only a few hours, which is not affected by transcription or translation inhibitors, and a *late-phase*, lasting more than 3-4h, which is affected by both (Flexner et al., 1963; Agranoff et al., 1965; McGaugh, 1966; Flexner et al., 1967; Davis and Squire, 1984; Krug et al., 1984; Stanton and Sarvey, 1984; recently reviewed by Abraham and Williams, 2008; Alberini, 2008; Hernandez and Abel, 2008; Klann and Sweatt, 2008; Costa-Mattioli et al., 2009). An intermediate phase of LTP that requires translation but not transcription has also been identified (Raymond et al., 2000; Kelleher et al., 2004a).

1.2. The synaptic tagging and capture (STC) hypothesis

The dependence of input-specific late-phase synaptic plasticity on transcriptional and translational processes implies the existence of a mechanism by which gene products required for the stabilisation of synaptic modifications, being synthesised in somatic or dendritic compartments (Martin et al., 2000a; Steward and Schuman, 2001; Wang and Tiedge, 2004; Martin and Zukin, 2006), are localized to stimulated synapses. Different hypotheses have been put forward on how this might be achieved (reviewed by Frey and Morris, 1998a; Jiang and Schuman, 2002; Kelleher et al., 2004a; Pang and Lu, 2004):

(i) the *mRNA targeting hypothesis* or *mail hypothesis* postulates that newly synthesised mRNAs encoding for relevant proteins are targeted to activated synapses, with no further need for localized activation of translational machinery;

(ii) the *local protein synthesis hypothesis* proposes that activated synapses synthesise and use the required proteins locally, with no need for mRNA targeting;

(iii) the *plasticity-factors or sensitization hypothesis* postulates that plasticity-related macromolecules are distributed to all synapses and that their general availability dictates the threshold by which synaptic activation produces long-lasting synaptic modifications. When many macromolecules are made available, e.g. following strong synaptic activation, the cell-wide threshold for induction of late-phase synaptic plasticity would be significantly reduced;

(iv) the *synaptic tagging and capture (STC) hypothesis* (Frey and Morris, 1997; Martin et al., 1997; Frey and Morris, 1998b; Kelleher et al., 2004a) attributes input-specificity of late-phase synaptic plasticity to two temporally and mechanistically dissociable events. The first event entails the transient, post-translational, *tagging* of synapses activated above a threshold during the induction of synaptic plasticity. The second event comprises the separate production and time-restricted diffused distribution of plasticity-related proteins along dendritic compartments. Transiently tagged synapses, but not neighbouring synapses, would be able to capture and use plasticity-related proteins, with no need for synapse-specific mRNA/protein targeting.

Evidence supporting the STC hypothesis was first obtained with the observation that protein synthesis (PS)-dependent late-phase synaptic plasticity can be induced during the absence of protein synthesis in rat hippocampal slices (Frey and Morris, 1997). This study used the typical two-pathway preparation, with stimulation of independent synaptic inputs to the same population of CA1 pyramidal neurons, to characterize a new form of long-term heterosynaptic facilitation of L-LTP. Repeated tetanisation of one input was first shown to result in the induction of homosynaptic PS-dependent L-LTP. Paradoxically, repeated tetanisation of the second input one hour later, during protein synthesis inhibition (i.e. superfusion of slices with the broad-spectrum translation inhibitor anisomycin), was also shown to result in homosynaptic L-LTP ("strong-before-strong" paradigm; Frey and Morris, 1997). This first result suggested that the proteins up-regulated by the stimulation of the first input stabilised the synaptic modifications triggered by the second input and was difficult to reconcile with both the mail and local synthesis hypotheses; given the requirement for coincident attribution of synaptic-specificity and protein synthesis at the time of induction these two hypotheses would predict that only the first input would sustain input-specific L-LTP. In agreement with this interpretation, a second series of experiments further showed that the delivery of a single tetanus to the second input (which on its own would induce only PS-independent E-LTP) less than 2.5-3h after (“strong-before-weak” paradigm), or even 1-2h before (“weak-before-strong” paradigm), the establishment of L-LTP in the first input, would result in PS-dependent L-LTP (Frey and Morris, 1997, 1998b). Overall, these results supported that the plasticity-related proteins up-regulated by the induction of L-LTP were sufficient to convert E-LTP to L-LTP and that the induction of both L-LTP and E-LTP established PS-independent synaptic tags. Equally difficult to reconcile with both the mail and local synthesis hypotheses, the important observation of a “weak-before-strong” heterosynaptic facilitation rendered the plasticity-factors hypothesis unlikely, as this hypothesis would require strong tetanisation to occur previous to the facilitatory effect (Frey and Morris, 1998b). Finally, a recent study has provided strong evidence in favour of the STC hypothesis and against the mail hypothesis. In this study Okada and

colleagues (Okada et al., 2009) found that elevation of intracellular cAMP levels in cultured rat hippocampal neurons resulted in the distribution of green fluorescent protein (GFP)-tagged Homer 1a (H1a) protein, a late-phase plasticity related protein synthesised in the soma, to dendrites but not to spines. The tagged protein was kept in the dendrites unless NMDA receptors were pharmacologically activated; in this case input-specific spine entry would occur mediated by dendritic vesicular transport. Overall these results suggest a mechanism for activity-dependent spine sorting of plasticity-related proteins synthesised in the soma which precludes the requirement for predetermined spine destination. Also difficult to reconcile with the mail hypothesis is the input-specificity of transcription-independent phases of late-phase synaptic plasticity; as noted somewhere else (Kelleher et al., 2004a), targeting of preexisting mRNAs would not be possible before synaptic activation.

Although the local synthesis hypothesis cannot explain the above-described evidence supporting heterosynaptic capture of plasticity-related proteins, local protein synthesis mechanisms and STC mechanisms are not mutually exclusive. Translational machinery is found in dendrites (Steward and Levy, 1982; reviewed by Martin et al., 2000a; Steward and Schuman, 2001, 2003; Wang and Tiedge, 2004; Martin and Zukin, 2006) and may even be localized to individual synaptic spines (Aakalu et al., 2001; Ostroff et al., 2002; Bourne et al., 2007) of rat hippocampal neurons. Dendritic translation is also known to play an important role in hippocampal late-phase synaptic plasticity (reviewed by Jiang and Schuman, 2002; Richter and Lorenz, 2002; Tang and Schuman, 2002; Martin, 2004; Sutton and Schuman, 2005; Schuman et al., 2006; Bramham and Wells, 2007; Bramham, 2008). It is possible that *(i)* local translational mechanisms are sufficient to support input-specificity of late-phase synaptic plasticity when synaptic activation is strong enough to trigger effective translation (e.g. Wang et al., 2009) but that, in addition, *(ii)* both strong and weak synaptic activation recruit synaptic tagging mechanisms that enable long-term synapse-specific associative phenomena. Intracellular cascades involved in recruiting and mediating local translation may contribute to tagging if they

are upstream of effective translation. In other words, a *partial* activation of local translational machinery, that would not result in effective translation but would *tag* activated synapses, would be able to explain input-specificity of L-LTP obtained in weakly tetanised inputs and inputs tetanised strongly during translational arrest. This would also provide a biochemically economical solution for the cell when having to respond to weak or strong activation differentially.

The generality of synaptic tagging and capture mechanisms to other species and neural systems has been suggested by studies focusing on branch-specific facilitation mechanisms of sensory to motor synapses in *Aplysia*. Briefly, in sensory-motor neuronal cultures, a single bifurcated *Aplysia* sensory neuron can establish connections with two spatially separated motor neurons. Delivery of a single pulse of serotonin (5-HT) to one of these branches induces short-term facilitation (STF) of synaptic efficiency lasting about 90min, while 5 spaced 5-HT pulses induce transcription- and translation-dependent, branch-specific, long-term facilitation (LTF), that can persist for more than 24h (Montarolo et al., 1986; Martin et al., 1997). In parallel to the above-described work in rodents, Martin and colleagues (1997) discovered that the delivery of a single pulse of 5-HT to one branch produced LTF if preceded by the delivery of 5 pulses to the other branch. Similarly to the experiments in rat hippocampal slices, LTF could only be "captured" within a specific time window. That is, if the single pulse was applied 1-4h *after* the 5 pulses or, importantly, 1-2h *before* the 5 pulses (Martin et al., 1997; Casadio et al., 1999; reviewed by Martin, 2002; Martin and Kosik, 2002; Barco et al., 2008).

These seminal studies in the rat hippocampus and in *Aplysia* were crucial not only because they provided strong support to the synaptic tagging and capture hypothesis but also because they broadened the traditional views on two important properties of synaptic plasticity, i.e. persistence and associativity. *First*, it was established that the same pattern of stimulation could produce either E-LTP or L-LTP depending on the history of activation of the neuron. This "variable persistence" (Frey and Morris, 1998a) of synaptic plasticity added to the view of a

fixed persistence mostly dictated by the strength of tetanisation. *Second*, it was shown that the associative properties of synaptic plasticity were not restricted to synaptic integration over the scale of milliseconds to seconds, attributed to NMDA-mediated coincidence detection mechanisms, but that they could also extend to minutes or even hours (Frey and Morris, 1997). The discovery of this new feature of synaptic plasticity, later coined "late-associativity" (Reymann and Frey, 2007), was particularly important because, as it will be addressed below, it may have important implications for associative learning and memory. Finally, these findings also shed a new perspective on the temporal nature of events required to establish long-lasting modifications of synaptic efficiency. Beyond the traditional view of unidirectional *sequential events* in which strong synaptic activation led to gene expression and consequently to synaptic consolidation, the synaptic tagging and capture hypothesis introduced the concept of bi-directional *additive events*, in which tagging and gene expression interact with each other to sustain input-specific synaptic consolidation (Barco et al., 2008).

1.2.1. Properties and mechanisms of synaptic tagging and capture *in vitro*

The potential of the synaptic tagging and capture hypothesis as an explanatory model for input-specificity of late-phase synaptic plasticity triggered a considerable number of studies investigating its properties and mechanisms in the CA1 hippocampal region *in vitro*. These studies expanded the original model to include important integrative properties such as cross-capture, transcompartmental-capture, tag-resetting and competitive maintenance, and have recently started to unveil some of the molecular substrates associated to putative tagging mechanisms (recent reviews by Reymann and Frey, 2007; Barco et al., 2008). They will now be addressed.

Cross-capture

"Capture" of late-phase synaptic plasticity, i.e. capture of plasticity-related proteins enabling stabilisation of synaptic efficiency changes, can be observed at depressed synapses as well as at potentiated synapses. Similar to the experiments described above in rat hippocampal slices focusing on L-LTP, later experiments focusing on L-LTD showed that the administration of a strong low-frequency stimulus to one input elicited transcription- and translation-dependent L-LTD that could be captured by the subthreshold low-frequency stimulation of a second input to the same population of CA1 hippocampal neurons (Kauderer and Kandel, 2000; Navakkode et al., 2004, 2005; Sajikumar et al., 2005). Interestingly, "cross-tagging" (Sajikumar and Frey, 2004; Sajikumar et al., 2005), or more correctly "cross-capture" (Govindarajan et al., 2006; Morris, 2006) can be observed between LTP and LTD mechanisms. In this phenomenon, establishment of L-LTP or L-LTD in one "weakly" stimulated input can be facilitated by the induction of the opposite form of late-phase synaptic plasticity in a second "strongly" stimulated input to the same population of neurons. Although this is not observed with branch-specific facilitation and depression in *Aplysia* (Guan et al., 2002), these results suggest that, at least in rat hippocampal CA1 synapses, L-LTP and L-LTD inducing stimulation recruit a common genetic pool and that different tags determine the stabilisation of either LTP or LTD in different synapses. Importantly, this mechanism may allow for the simultaneous stabilisation of bidirectional plasticity (Govindarajan et al., 2006), which may be critical for efficient memory storage (Bear, 1996).

Compartment-specific tagging and trans-compartmental capture

Pyramidal neurons in the CA1 hippocampal region receive different types of information from other hippocampal and extra-hippocampal regions through apical and basal dendrites (Amaral and Lavenex, 2007). In a recent set of experiments, Alarcon and colleagues (2006)

showed that capture of L-LTP could be observed not only in apical dendrites, where it had been originally described (Frey and Morris, 1997), but also in basilar dendrites. Furthermore, capture of L-LTP was observed to occur across basilar and apical dendritic compartments provided that the stimulation of the "weak" input was strong enough, i.e. a single train of high-frequency stimulation (100Hz) was sufficient to capture L-LTP within compartments but capture across compartments required two trains of high-frequency stimulation. The fact that the same stimulation protocol that captured L-LTP within compartments failed to capture L-LTP across compartments suggested to the authors that synaptic tagging was compartment-specific. Later studies supported this idea by showing that late-phase synaptic plasticity induced in basilar or apical dendritic compartments did not spread across compartments and that input-specificity of synaptic plasticity may be determined by activation of compartment-specific tagging molecules (see below; Sajikumar et al., 2007a). Mechanisms of compartment-specific tagging and cross-capture have been proposed to enable the integration of information and formation of memory traces at the level of functional, independent, synaptic populations and are thought to be advantageous from the point of view of computational capability of individual neurons and neural networks (Govindarajan et al., 2006; Sajikumar et al., 2007a). Considering that compartmentalised and non-compartmentalised capture may allow individual neurons to integrate information differentially as a function of stimulus strength (Alarcon et al., 2006), Frey and colleagues have further speculated that non-stressful cognitive behaviours may be processed by compartment-specific plasticity depending on local translation, whereas stressful life-events, with a high emotional content and requiring storage of information in a larger number of neuronal networks, would induce cell-wide transcription-dependent up-regulation of protein synthesis (Reymann and Frey, 2007; Sajikumar et al., 2007a; Sajikumar et al., 2007b).

Tag resetting

As mentioned above, the tag is only transiently active, with an approximate lifetime of about 1-2h in both rat hippocampal synapses (Frey and Morris, 1998b) and *Aplysia* sensory-motor synapses (Casadio et al., 1999) *in vitro*. While "deactivation" of synaptic tags may occur *passively* by cellular degradational processes, some studies suggest that it may also be *actively* mediated by yet unknown regulatory mechanisms that are activated in response to specific patterns of synaptic stimulation closely after induction of LTP. Sajikumar and colleagues (2004b) first shown that low-frequency stimulation inducing depotentiation (DP) *resets* synaptic tags when applied 5-10min after their setting (see also Sajikumar et al., 2009). Later studies also revealed that DP-inducing stimulation applied homosynaptically or heterosynaptically before L-LTP induction had no effect on synaptic capture by subsequent E-LTP stimulation but that applied just before E-LTP prevented its conversion to L-LTP (Young and Nguyen, 2005). These findings suggest that compartment-specific tagging (Alarcon et al., 2006; Sajikumar et al., 2007a) can be regulated by transcription-independent cell-wide mechanisms.

Competitive maintenance

It was recently shown that, under an artificially induced regime of reduced protein synthesis in rat hippocampal slices, induction of L-LTP in one input to CA1 pyramidal neurons disrupted the maintenance of pre-established L-LTP in a second independent input to those same cells (Fonseca et al., 2004). These results add to the synaptic tagging and capture hypothesis by suggesting that, under specific circumstances, plasticity-related proteins can also be captured from previously stabilised synapses in a competitive fashion, a phenomenon termed as *competitive maintenance* (Fonseca et al., 2004). Capture may, therefore, be a reversible process whose cooperative or competitive nature is determined by the general availability of plasticity-related proteins.

Tagging mechanisms

Based on the above described studies, candidate mechanisms for synaptic tagging should fulfil a number of specific criteria (Martin and Kosik, 2002; Kelleher et al., 2004a): *First*, they should be restricted to single synapses; *second*, they should be protein synthesis-independent; *third*, they should be induced by early- and late-phase LTP or LTD; *fourth*, they should be transient (1-2h) and susceptible to resetting; *finally*, they should be able to interact with mRNAs and/or proteins. In view of this, a mechanism that would be simultaneously involved in E-LTP or E-LTD, tag-setting and capture of plasticity-related proteins, would also be economically advantageous to the cell (Frey and Morris, 1998a).

Tagging is assumed to depend on the activation of NMDA receptors, however, the fact that blocking this type of receptor also blocks the induction of E-LTP makes it difficult to provide conclusive evidence that it occurs downstream NMDA-receptor activation (O'Carroll and Morris, 2004). Mechanisms proposed to comply with the above-described requirements for tagging include: the activation of specific protein kinases (Frey and Morris, 1998b; Barco et al., 2002; Navakkode et al., 2004; Sajikumar et al., 2005; Alarcon et al., 2006; Huang et al., 2006; Young et al., 2006; Sajikumar et al., 2007a; Sajikumar et al., 2009); the transient insertion of AMPA receptors in the postsynaptic membrane (Frey and Morris, 1998b; Carroll and Malenka, 2000; Plant et al., 2006; Matsuo et al., 2008); the activation of postsynaptic TrkB receptors (Barco et al., 2005; Lu et al., 2008); activation of local translational machinery (Barco et al., 2008); actin polymerization (Bramham and Wells, 2007); modifications of the spine cytoskeleton (Frey and Morris, 1998b; Sanchez et al., 2000; Luo, 2002); and, potentially related to several of the above, activity-dependent regulation of protein spine entry (Okada et al., 2009).

Particular attention has been given to the possibility that tagging may be mediated by protein kinase activity (Frey and Morris, 1998b). The fact that the time course of protein phosphorylation falls within the expected lifetime of the tag and that activity-regulated kinases can modulate early-phase synaptic plasticity phenomena, as well as have their activity restricted

by membrane anchoring, supports this idea (Barco et al., 2008). Also, evidence suggesting the involvement of cAMP-dependent protein kinase A (PKA), the calcium/calmodulin-dependent protein kinase II (CAMKII), PKMζ and the extracellular signal-regulated kinase 1/2 (ERK), in synaptic tagging mechanisms, has been recently provided (Barco et al., 2002; Navakkode et al., 2004; Sajikumar et al., 2005; Huang et al., 2006; Young et al., 2006; Sajikumar et al., 2007a; Sajikumar et al., 2009). Interestingly, the participation of protein kinases in synaptic tagging seems to be both process-specific and compartment-specific (Sajikumar et al., 2007a). Process-specificity refers to the type of plasticity induced, i.e. LTP or LTD. In view of this, activation of PKA, CAMKII and PKMζ, has been reported to participate in tagging of LTP (Barco et al., 2002; Sajikumar et al., 2005; Young et al., 2006; Sajikumar et al., 2007a; Sajikumar et al., 2009), whereas activation of ERK has been reported to contribute to tagging of LTD (Sajikumar et al., 2007a). In what concerns compartment-specificity, it has been proposed that tagging of LTP is mediated by CAMKII in apical dendrites and PKA and PKMζ in basal dendrites (Sajikumar et al., 2007a). Other studies, however, have implicated PKA activation in tagging of LTP in apical dendrites (Alarcon et al., 2006; Young et al., 2006). Finally, two recent studies strongly support the idea that CAMKII may play an important role in tagging LTP (Sajikumar et al., 2007a). First, Sajikumar and colleagues (2009) showed that activation of ryanodine receptors primes subthreshold stimulation to tag LTP and that this mechanism is mediated by activation of CAMKII (Sajikumar et al., 2009). Second, Lee and colleagues (2009) showed that the induction of LTP and associated spine enlargement triggers the transient activation of CAMKII restricted to stimulated spines. Activation of CAMKII in spines, following glutamate uncaging and depolarization, depended on NMDA receptor activation (Lee et al., 2009). This later study suggests a mechanism by which NMDA receptor activation, cytoskeleton modifications and transient protein kinase activity may be recruited concertedly during LTP induction to tag activated synapses. As a last note, it is important to refer that neuromodulatory receptors such as dopaminergic receptors, β-adrenergic receptors and muscarinic acetylcholine

receptors, are thought to couple to some of these protein kinases, such as PKA and ERK, to up-regulate protein synthesis and stabilise NMDA-dependent LTP in the CA1 hippocampal region (Frey et al., 1993; Abel et al., 1997; Nayak et al., 1998; Roberson et al., 1999; Nguyen and Woo, 2003; Banko et al., 2004; Kelleher et al., 2004b; Kelleher et al., 2004a; Gelinas et al., 2007; Gobert et al., 2008). This suggests that some protein kinases may contribute to both process-specific tagging and process-non-specific up-regulation of protein synthesis.

There is some evidence suggesting that the up-regulation of plasticity-related proteins required for heterosynaptic L-LTP facilitation in the CA1 hippocampal region depends on the co-activation of glutamatergic and neuromodulatory dopaminergic inputs, and more specifically, on the co-activation of glutamate NMDA receptors and dopamine D1/D5 receptors (O'Carroll and Morris, 2004; Sajikumar and Frey, 2004). Little, however, is known about the identity of the mRNAs or proteins required to stabilise synaptic plasticity at tagged synapses and about the nature of the capture mechanisms. The few existing studies suggest that BDNF (Pang and Lu, 2004; Pang et al., 2004; Barco et al., 2005; Barco et al., 2008; Lu et al., 2008) and PKMζ (Sajikumar et al., 2009) may be important plasticity-related proteins required to stabilise LTP heterosynaptically (see also Okada et al., 2009). Capture mechanisms may involve phosphorylation and synaptic incorporation of plasticity-related proteins, the local translation of diffusely transported mRNAs, or other mechanisms, but no conclusive evidence has been provided supporting any of these possibilities (Barco et al., 2008; Lu et al., 2008; but see Okada et al., 2009).

1.2.2. Behavioural implications

Despite the proliferation of studies characterizing the properties and mechanisms of synaptic tagging and capture *in vitro*, as well as integrating synaptic tagging and capture properties in computational models of synaptic plasticity and memory formation (Chialvo and

Bak, 1999; Smolen et al., 2006; Smolen, 2007; Clopath et al., 2008; Barrett et al., 2009), two essential questions for the physiological relevance of this phenomenon remain unanswered. *First*, do STC mechanisms occur *in vivo*? *Second*, do STC mechanisms contribute to the formation of memory?

It is surprising that, more than a decade after the seminal papers on the synaptic tagging and capture hypothesis were published, there has not yet been any report of success (or failure) to detect synaptic tagging and capture mechanisms in two pathway experiments in anaesthetized or freely moving animals. This may be due to the technical demands of such experiment (Hassan et al., 2006). Indeed, stable recordings after induction of early-phase and/or late-phase NMDA-dependent synaptic plasticity in two independent pathways projecting to the same population of neurons in living animals are not easy to achieve. Alternatively, some efforts have been made to show reinforcement of LTP *in vivo* following electrically- or behaviourally-induced recruitment of heterosynaptic neuromodulatory inputs.

LTP-reinforcement studies focused on noradrenaline-, acetylcholine-, dopamine- and glucocorticoid-mediated signalling mechanisms that have been widely implicated in the consolidation of both synaptic plasticity and memory in the hippocampus (reviewed by Bailey et al., 2000; McGaugh, 2000; McGaugh and Roozendaal, 2002; Jay, 2003; Wise, 2004; Lisman and Grace, 2005; Diamond et al., 2007; Sara, 2009). Interest in heterosynaptic dopaminergic modulation was also evidently driven by its involvement in up-regulation of protein synthesis (Smith et al., 2005) and synaptic tagging and capture mechanisms (O'Carroll and Morris, 2004; Sajikumar and Frey, 2004) in the hippocampus. As reviewed by Reymann and Frey (2007), these studies investigated three different types of phenomena:

First, "structural reinforcement" was investigated in studies assessing the effects of associative electrical stimulation of neuromodulatory brain structures, such as the basolateral amygdala, the ventral tegmental area, and others, on the stabilisation of E-LTP in the hippocampal DG and CA1 regions of freely moving animals;

Second, "emotional tagging", a term firstly introduced by Arikav and Richter (2003), was investigated in studies addressing the effects of emotional arousal (such as that related to moderate stress or novelty detection), which facilitates the rapid consolidation of new information in the hippocampus (e.g. Lisman and Grace, 2005; Akirav and Richter-Levin, 2006; Diamond et al., 2007; see below), on the stabilisation of transient forms of synaptic plasticity in those same hippocampal regions *in vivo*;

Third, "cognitive tagging", was investigated in studies testing the possibility that other *less-emotional* cognitive contents, e.g. associated to repetitive learning (see Korz and Frey, 2004; Uzakov et al., 2005), would also stabilise hippocampal synaptic plasticity in freely moving animals.

Although synaptic tagging and capture mechanisms have been, up to the present moment, exclusively characterized in the CA1 hippocampal region, most of the studies investigating structural and behavioural reinforcement have focused on synaptic plasticity in the dentate gyrus. In a pivotal study, activation of the basolateral amygdala (BLA), which is known to facilitate the consolidation of hippocampal-dependent memory (reviewed by McGaugh, 2000; Richter-Levin and Akirav, 2000; Pare, 2003; Akirav and Richter-Levin, 2006; LaBar and Cabeza, 2006; Tsoory et al., 2008), was shown to convert E-LTP (~4h) to L-LTP (~8h) in the DG when administered 5-30min before, or 5-15min after, tetanisation of the perforant path in freely moving rats (Frey et al., 2001). Intraventricular administration of glutamate NMDA-receptor and dopamine D1-receptor antagonists between E-LTP induction and BLA stimulation (when separated 15min apart) did not alter the time course of the BLA-reinforced LTP. BLA-reinforcement (BLAR) was, however, dependent on translation and on the activation of acetylcholine muscarinic and noradrenergic β-adrenergic receptors. As BLA does not project directly to the DG (Pikkarainen et al., 1999; Pitkanen et al., 2000) and drugs were administrated intraventricularly these results were not interpretable concerning the site of drug action; this could have been the BLA, the hippocampus or relay structures between these two brain regions. In later studies by the same group, stimulation of the medial septum, the main cholinergic input to the hippocampus (Vizi and Kiss, 1998), 15min after tetanisation of the perforant-path was also shown to reinforce E-LTP into L-LTP in the DG (Frey et al., 2003) (see also Jas et al.,

2000). This suggested that the septal-hippocampal pathway could mediate BLAR. As in BLAR, septal-reinforcement was shown to depend on β-adrenergic receptor activation and protein synthesis. Curiously, activation of acetylcholine muscarinergic receptors was not required (see also Almaguer-Melian et al., 2005). However, this study also comprised intraventricular administration of drugs and was open to the same line of criticism described above. A more recent study addressed this issue (Bergado et al., 2007). In these experiments, administration of drugs between induction of LTP and stimulation of the BLA was targeted to the specific regions of interest, namely, the BLA, the DG, the medial septum (MS) and the locus coeruleus [LC; the main noradrenergic input to the hippocampus (Vizi and Kiss, 1998)], during BLAR. Local infusion of atropine (*a muscarinergic antagonist*) or propanolol (*a β-adrenergic receptor antagonist*) into the DG, lidocaine (*a local anaesthetic*) or propanolol into the MS, and lidocaine or atropine into the LC, impaired BLAR. Infusions of atropine or propanolol into the BLA did not interfere with BLAR. Overall, these results suggest that BLAR is mediated by activation of the MS and the LC and that it requires activation of noradrenergic and cholinergic projections to these structures, respectively. They also suggest that activation of noradrenergic and cholinergic projections to the DG, but not to the BLA, is required for BLAR; these may be projections from the LC and the MS. As the BLA does not project directly to any of these structures their recruitment during DG-LTP reinforcement must also be indirect (Pare, 2003; but see Bergado et al., 2007). These are important findings because they largely contribute to the better understanding of the mechanisms underlying the modulation of L-LTP in the hippocampus in freely moving animals and because they set a working model to investigate the putative neurophysiological substrates of long-term memory enhancement by emotional arousal (see below). Of concern to the synaptic tagging and capture hypothesis is that these studies have not investigated if heterosynaptic reinforcement of LTP in the dentate gyrus requires up-regulation of protein synthesis in that same brain region.

A recent unpublished study by Frey's group in Magdeburg (Scherf et al., FENS 2008) has further investigated the effects of high-frequency electrical stimulation of the ventral tegmental area [VTA; a brain structure which innervates the CA1 region with dopamine (Scatton et al., 1980)] on the stabilisation of E-LTP in the CA1 hippocampal region of freely moving rats. Activation of the VTA 15min after stimulation of the contralateral CA3 hippocampal region resulted in the stabilisation of CA1-LTP, which persisted over 24h. This effect was presumably mediated by activation of dopamine D1 receptors and protein synthesis, but this is yet to be published (see Reymann and Frey, 2007). If evidence were to be provided that up-regulation of protein synthesis in the CA1 hippocampal region is a requirement for VTA-reinforcement of LTP in that brain region, this would strongly support the existence of synaptic tagging and capture mechanisms *in vivo*.

The effects of emotional arousal on previously induced DG-LTP have been investigated following different experimental conditions such as water delivery after deprivation (Seidenbecher et al., 1995; Seidenbecher et al., 1997; Almaguer-Melian et al., 2006), delivery of a footshock (Seidenbecher et al., 1997), exploration of a novel environment (Xu et al., 1998; Abraham et al., 2002; Straube et al., 2003b; Straube et al., 2003a) and swim-induced stress (Korz and Frey, 2004; Sajikumar et al., 2007b). From these studies, weak support for a role of STC events in behavioural reinforcement of DG-LTP can be found in the work of Seidenbecher and colleagues (1995). In this study the authors showed that allowing water-deprived rats to drink after weak tetanic stimulation slightly prolonged unsaturated LTP, however, the weak increase is insufficient to conclude that E-LTP was transformed in L-LTP. Other studies investigating the exploration of novel environments and swim-induced stress have produced mixed results or do not allow distinguishing between STC and sensitisation-mediated mechanisms.

Beyond reinforcement studies, another point that has to be considered is whether or not STC mechanisms are involved in the formation of memory, as predicted by the STC hypothesis (Frey and Morris, 1997; Morris, 2006). Some distinctive features of the STC model, such as the involvement of neuromodulatory inputs involved in emotional processing (e.g. dopaminergic inputs), and the cellular integration and consolidation of different inputs within a period of time that scales from minutes to hours (i.e. late-associativity), suggest that tagging and capture mechanisms may participate in the formation of a specific type of memory initially referred to by Stratton as *retroactive hypermnesia* (Stratton, 1919), and later by Brown and Kulik as *flashbulb memory* (Brown and Kulik, 1977). In his seminal paper addressing the effects of emotion on memory recollection, Stratton (1919) used the testimony of some of his students to describe how certain life events that carried a strong emotional charge or intense surprise, such as the San Francisco earthquake of 1906, produced vivid long-term recollection of neutral "easily-forgettable" facts and episodes occurring before (up to the preceding day), or closely after (up to a few hours), those events. According to the author, these vivid recollections would most frequently relate to the pictorial aspect of the experience[1] (but not always), and would mostly be determined by the intensity rather than the quality of the emotional *excitement* [e.g. fear, fearless surprise, pleasurable surprise, amongst others (Stratton, 1919)]. To conclude his article he writes: *"... preceding experiences, especially those of hours past or of the preceding day, probably do not at the time of emotion exist in the form of actual images ... the emotion, then, would seem to have the power to go behind mere imagery into these dispositions or traces, and to strengthen them and the connections by which they may be called into life. And not only the traces of the experience which aroused the emotion, but also of trivial and neutral events antecedent to the emotion itself."* It is easy to conceive how STC mechanisms could contribute to the formation of this type of memory; plasticity-related proteins up-regulated by the

[1] It was on the account of being like one-shot photographs that these long-term memories for life events with a particular emotional or cognitive relevance were later addressed to as *flashbulb memories* (Brown and Kulik, 1977; see also Scott et al., 1996; Finkenauer et al., 1998; Sierra and Berrios, 1999; Diamond et al., 2007). According to Brown and Kulik (1977) the creation of flashbulb memories depended on a high level of "surprise", a high level of "consequentiality" (i.e. perceived relevance to the individual), and high level of "arousal".

"*experience that aroused the emotion*" would be captured and used by synapses activated by the "*neutral*" events to stabilise transient synaptic modifications underlying the memory traces for those same events. This would, of course, require that both *strong* "emotional" and *weak* "neutral" encoding events recruited an overlapping population of neurons. But can STC mechanisms be detected during the formation of memory?

There is now an extensive literature suggesting that emotional arousal facilitates the consolidation of memory for neutral events in both humans (e.g. Nielson et al., 2005; Anderson et al., 2006; McGaugh, 2006) and rodents (reviewed by Richter-Levin and Akirav, 2003; Akirav and Richter-Levin, 2006; Diamond et al., 2007). This phenomenon is thought to depend on the activation of β-adrenergic receptors in the amygdala and amygdala-mediated modulation of synaptic plasticity in structures involved in memory consolidation, such as the hippocampus. In view of this, and trying to establish a parallel with the STC hypothesis, Akirav and Richter-Levin (2003; 2006) proposed that the amygdala would *"mark"* or *"tag"* emotionally charged experiences as important by activating modulatory inputs and by strengthening synapses located on neurons that had been activated in other brain regions engaged in the learning situation - the "*emotional tagging*" hypothesis (Richter-Levin and Akirav, 2003). According to the authors, the heterosynaptic activation of modulatory transmitter systems could " … *reduce the threshold for the induction of the tag or for the activation of protein synthesis, or it could be crucial for the synthesis of plasticity proteins. Either way, it would support the transformation of early- into late-phase memory* " (Richter-Levin and Akirav, 2003). In a strict sense the term "*emotional tagging*" may be misleading as the setting of the tag *per se* may not contribute to the stabilisation of synaptic connections and effective up-regulation of plasticity-related proteins can be dissociated from tagging mechanisms. There is no direct evidence that STC mechanisms contribute to BLA-mediated consolidation of memories for trivial events; however, two recent studies suggest that emotional arousal may indeed stabilise hippocampal-dependent memories via STC mechanisms. During emotional arousal noradrenaline is released from both LC and

lateral brain stem tegmentum neurons and these project not only to the amygdala but also to the hippocampus and other brain regions (LeDoux, 2000; Maren, 2001; Fanselow and Gale, 2003). A recent study by Hu and colleagues (Hu et al., 2007) suggests that emotion enhances hippocampal-dependent memory via noradrenaline-mediated regulation of AMPA receptor trafficking to synapses. In support of this they first observed that noradrenergic signalling induces transient (up to 30min) phosphorylation of GluR1-containing AMPA receptors at sites that are critical for synaptic delivery (Ser845 and Ser831), in organotypic hippocampal slices. *Second*, phosphorylation of GluR1-AMPA receptors in the hippocampus was also observed *in vivo* following systemic injection of noradrenaline or emotional stress (induced in mice by exposure to fox urine). *Third*, noradrenaline facilitated the induction of LTP in hippocampal slices by subthreshold stimulation when applied up to 30min before tetanisation (the same time window observed *in vitro* for GluR1-AMPA receptor phosphorylation). *Fourth*, noradrenaline facilitated the synaptic delivery of GluR1-AMPA receptors in the hippocampus. *Fifth*, noradrenaline-mediated facilitation of LTP was defective in mice in which Ser845 and Ser831 were mutated to prevent phosphorylation. *Finally*, systematic injection of noradrenaline facilitated subsequent contextual fear conditioning in wild–type, but not mutant, mice. A second study (Matsuo et al., 2008) has now shown selective recruitment of newly synthesised green fluorescent protein (GFP)-tagged GluR1-AMPA receptors to mushroom spines of hippocampal CA1 neurons *in vivo* after contextual fear conditioning. The fact that delivery of GFP-GluR1 receptors to spines occurred only a few hours after training suggested to the authors that at the time of learning there were changes in specific spines that allowed the capture of newly synthesised AMPA receptors at later points. Overall, the results from these two studies suggest that: *1)* contextual fear conditioning sets "behavioural tags"; *2)* GluR1-containing AMPA receptors may be one of the cargo molecules selectively delivered to tagged synapses; and finally, *3)* Emotional arousal may provide that cargo to facilitate the formation of long-term memory for contextual fear conditioning.

To establish that STC mechanisms contribute to the formation of memory, it is necessary to show the stabilisation of a memory for an experience that - ordinarily - would only produce short-lasting memory. Such stabilisation would happen if that experience was followed or, as also anticipated by the STC hypothesis, preceded by a second experience that up-regulates protein synthesis in cells activated by both experiences. A more strict interpretation of the STC hypothesis, one that would provide a more adequate model for the formation of *flashbulb memories*, would in addition assume that different memories would be formed for each experience and that encoding of these memories would recruit independent but overlapping populations of neurons; up-regulation of protein synthesis would, in this case, occur in a brain region underlying consolidation of memory for both experiences. As referred above, for any of these interpretations it would be crucial to show a "weak-before-strong" version of the paradigm, to discard interpretations based on the sensitization hypothesis.

Three different studies have directly investigated the contribution of synaptic tagging and capture mechanisms to the formation of memory. In a first unpublished study, Jerry Rudy and colleagues (Rudy et al., SFN 2005) investigated if long-term (24h) memory for *weak* auditory-cue and contextual fear conditioning elicited by a weak shock (0.6mA) could be enhanced by a strong electrical shock (1.5mA) in a different context delivered before or after CS-US pairing. The strong electrical shock enhanced tone conditioned freezing when given 90min but not 6h before, or 15min but not 6h after, CS-US pairing. Contextual fear conditioning was also enhanced when the strong shock was administered 15min after CS-US pairing, which was shown not to result from the generalization of the strong shock to the conditioning context. These were interesting results as they resembled the symmetry observed in "strong-before-weak" and "weak-before-strong" two-pathway experiments in rat hippocampal slices (Frey and Morris, 1997, 1998b), however, pharmacological evidence showing that the rescuing effect was mediated by protein synthesis in relevant brain regions is yet to be provided. As in fear conditioning the hippocampus is generally thought to encode a representation of the context

with the associative changes for fear occurring in the amygdala (Anagnostaras et al., 2001), this should be a considered target in these experiments. A second, more complete study, investigated tagging and capture mechanisms during inhibitory avoidance learning (Moncada and Viola, 2007). In this study, the authors found that memory for *weak* inhibitory avoidance (IA) training, which ordinarily induces short-term memory (15min) but not long-term memory (24h), could be consolidated into long-term memory by the exploration of a novel environment, but not familiar one, occurring 1h before, or 15min after, the training session. The novelty effect was dependent on protein synthesis, as infusion of anisomycin in the dorsal hippocampus immediately after the pretraining exposure to novelty blocked the formation of long-term memory. Novelty-induced consolidation of memory was also shown to depend on the activation of dopamine D1/D5 receptors in the hippocampus. Overall, these results suggest that exploration of the novel environment recruits heterosynaptic dopaminergic signalling to up-regulate the plasticity-related proteins required to stabilize the IA memory trace at tagged synapses. This is strongly suggestive, but still indirect evidence, that STC mechanisms occur *in vivo* and contribute to the formation of memory. Worth noticing is the fact that up-regulation of protein synthesis was not associated to the formation of long-term memory for its triggering event. Finally, a third study investigated behavioural tagging mechanisms using latent inhibition of conditioned taste aversion (Merhav and Rosenblum, 2008). During conditioned taste aversion (CTA) an organism learns to avoid specific tastes associated to sickness. In latent inhibition of CTA the animals are pre-exposed to a novel taste a few days before CTA and this pre-exposure weakens the aversion to that same taste, implying formation of taste memory. Taste-memory consolidation depends on protein synthesis in the gustatory cortex. In this study the authors investigated if memory for a *weak* taste input (producing short-lasting memory) would be stabilised when this input was preceded by a different *strong* taste input (producing longer-lasting memory) during the pre-exposure day. They also investigated if a strong taste input would rescue memory for a different strong input occurring during anisomycin-mediated translational arrest in the gustatory cortex. Pre-exposures took place 2 or 3 days before conditioning and retention of memory was assessed

2 days after conditioning. A novel, but not a familiar, strong input facilitated the formation of long-term memory for the weak input (when given 30min to 5h apart), however, it was unable to rescue memory for the second strong input occurring 100min later, under protein synthesis inhibition. Considering these results the authors suggested that the interaction between strong and weak inputs might have been mediated by novelty-triggered long-term neuromodulatory activity rather than up-regulation and capture of plasticity-related proteins. Although the Moncada and Viola (2007) study provided some evidence that STC mechanisms may occur *in vivo* and contribute to memory formation, there are still several open questions. For example, can behavioural tagging be detected only in the formation of specific types of memory? Are STC mechanisms restricted to the hippocampus in mammals? Is the effective behavioural up-regulation of protein synthesis restricted to strong novelty events?

1.3. Aims of the thesis

The main objective of this thesis is to investigate whether, as predicted by the STC hypothesis, STC mechanisms can be detected during the formation of memory, and specifically, during the formation of rapidly acquired allocentric place memory. The focus on rapidly acquired allocentric place memory is for the following reasons:

First, in the mammalian brain, STC mechanisms have been, up to this moment, exclusively characterized as properties of hippocampal NMDA-dependent synaptic plasticity (Frey and Morris, 1998b; O'Carroll and Morris, 2004; Sajikumar and Frey, 2004; Reymann and Frey, 2007). The important role of the hippocampus and of hippocampal NMDA-mediated mechanisms in the formation of both reference and rapidly acquired allocentric place memory has been widely documented (Morris et al., 1982; Morris et al., 1986; Eichenbaum et al., 1990; Moser et al., 1995; Tsien et al., 1996; Riedel et al., 1999; Steele and Morris, 1999; Ferbinteanu et al., 2003; Nakazawa et al., 2003; Nakazawa et al., 2004; de Hoz et al., 2005). Furthermore, there is also some evidence suggesting that encoding of allocentric place memory is mediated by NMDA-dependent synaptic plasticity in the hippocampus (e.g. Moser et al., 1998).

Second, there is a vast literature suggesting that long-term allocentric place memory relies on mechanisms sustaining late-phase NMDA-dependent synaptic plasticity in the hippocampus (e.g. Guzowski and McGaugh, 1997; Kogan et al., 1997; Meiri and Rosenblum, 1998; Guzowski et al., 2000; Guzowski et al., 2001; Gusev et al., 2005; Plath et al., 2006; McGauran et al., 2008).

Third, some of the requirements for synaptic tagging and capture mechanisms in the hippocampus, such as dopaminergic heterosynaptic neuromodulation have also been shown to play an important role in the stabilisation of rapidly acquired allocentric place memory (O'Carroll and Morris, 2004; Sajikumar and Frey, 2004; O'Carroll et al., 2006).

Fourth, studies investigating the implications of the synaptic tagging and capture hypothesis for the formation of memory have focused on learning paradigms such as fear conditioning (J. Rudy et al.; *unpublished*), inhibitory avoidance (Moncada and Viola, 2007), and taste aversion (Merhav and Rosenblum, 2008). It is important to test the generality of this phenomenon to other types of memory and behavioural paradigms.

The main question addressed in this thesis can be formulated as follows:

Assuming that synaptic plasticity phenomena underlie learning and memory (Bliss and Lomo, 1973; Bliss and Collingridge, 1993; Martin et al., 2000b; Martin and Morris, 2002) and that STC mechanisms occur *in vivo,* can these mechanisms be detected during the formation of place memory so that *a short-lasting place memory becomes long-lasting when its encoding event (target event) is preceded, or followed, within a limited time window, by a separate encoding event (modulatory event) which produces a different long-lasting protein synthesis-dependent place memory, and up-regulates plasticity-related proteins in cells encoding memory traces for both events?*

The first series of experiments presented in this thesis address experimental requirements to test this hypothesis. Briefly, in these experiments, two new behavioural tasks were developed which provided sensitive measures of rapidly acquired allocentric place memory – the new one-trial place memory task in the "event arena" (EXPT. 1-4) and the new delayed matching-to-place (DMP) protocol in the watermaze (EXPT. 8-10). Using these tasks, which differ as to the nature of motivation and navigational demands, persistence of both aversively- and appetitively-motivated allocentric place memory for a single acquisition trial was determined and compared (EXPT. 5 AND 8-10). Given the putative role of NMDA receptor activation in STC mechanisms, the contribution of NMDA and AMPA receptor activation in the hippocampus during encoding and retrieval of rapidly acquired allocentric place memory (one-trial memory) was also investigated (EXPT. 6-7; in the "event arena" task). Finally, in order to characterise "weak" and "strong" encoding events leading to the formation of either short- or long-lasting allocentric place memory, a series of experiments investigated memory strength for different types of encoding events in the watermaze. Here, memory for a single acquisition trial, massed multi-trial training, and spaced multi-trial training was assessed and compared (EXPT. 11-13). An additional experiment (EXPT. 14) established that 3 spaced "standard" trials (10min ITI), in which rats were allowed to swim to the platform, produced strong memory detectable 24h after

training (strong encoding event), whereas 3 placement trials (10min ITI), in which animals were only placed in the platform for 30s, produced weak memory detectable 30min, but not 24h, after training (weak encoding event).

A second series of experiments tested the above-described hypothesis by investigating synergistic interactions between encoding events occurring in two different watermazes. First, weak and strong encoding events were combined to test behavioural analogues of the "strong-before-weak" and "weak-before-strong" STC paradigms characterised in electrophysiological experiments (EXPT. 15-17) (Frey and Morris, 1997, 1998b). Then, after establishing *i)* the time course and local specificity of protein synthesis inhibition by intra-hippocampal infusion of anisomycin *in vivo* (using [^{14}C] L-leucine uptake autoradiography) and *ii)* the dependence of long-term memory for strong encoding events on protein synthesis in the hippocampus (EXPT. 18-19), a behavioural analogue of the "strong-before-strong" STC paradigm (Frey and Morris, 1997) was investigated (EXPT. 20). Finally, catFISH (cellular compartment analysis of temporal activity by fluorescent *in situ* hybridization) and imunohistochemistry were used to determine the level of overlap of hippocampal neuronal ensembles activated by two strong encoding events occurring in different environments (different watermaze rooms), and to investigate up-regulation of mRNA and protein synthesis in the hippocampus following strong encoding events in the watermaze (EXPT. 21).

1.3.1. Development of behavioural tasks to study the persistence of rapidly acquired allocentric place memory (Expt. 1-5/8-10)

Place memory tests for rodents are important tools to reveal neurobiological substrates and psychological processes relevant to human declarative memory (Bures and Fenton, 2000; Aggleton and Pearce, 2001). Place and declarative memory rely on the rapid encoding of relations among multiple cues and share neurobiological substrates, with the hippocampus and

parahippocampal cortices playing key roles (O'Keefe and Nadel, 1978; Squire, 1992; Eichenbaum, 2000; Leutgeb et al., 2005). Two of the most widely used behavioural tasks to assess rapidly acquired allocentric place memory in rodents are the radial-arm maze task (Olton and Samuelson, 1976) and the delayed matching-to-place task in the watermaze (Morris, 1983, 1984; Panakhova et al., 1984; Whishaw, 1985; Steele and Morris, 1999).

In the radial-arm maze task rats are placed on a platform that is central to several (usually eight) radiating arms and have to retrieve food rewards that are only present once at the end of each arm (Olton and Samuelson, 1976). The rat has to rely on memory for the spatial location of visited arms relative to extra-maze landmarks (allocentric place memory) in order to retrieve new rewards and is always forced to return to the central platform before making another choice. A delay may be interposed between choices to study the persistence of memory for the location of previously retrieved rewards (Olton and Samuelson, 1976; Beatty and Shavalia, 1980; Buresova, 1980). Systematic studies of place memory persistence in this task have been controversial, with reported decay of memory varying from tens of minutes to many hours (Beatty and Shavalia, 1980; Buresova, 1980; Markowska et al., 1983; Maki et al., 1984; Bolhuis et al., 1986; Strijkstra and Bolhuis, 1987). Strain differences (Markowska et al., 1983), variations in apparatus (Maki et al., 1984) and training procedures (Markowska et al., 1983; Maki et al., 1984; Bolhuis et al., 1986; Strijkstra and Bolhuis, 1987), have been proposed as likely explanations for the observed discrepancies.

In the delayed matching-to-place (DMP) task in the watermaze (Morris, 1983, 1984; Panakhova et al., 1984; Whishaw, 1985; Steele and Morris, 1999), rats have to escape to a hidden platform that is moved to a new location after a few trials (usually between test days). As in delayed responses in the radial-arm maze task, escape efficiency depends on rapid acquisition and subsequent retrieval of allocentric place memory. Place learning in this task is usually measured as a reduction in escape latencies or in path lengths across successive trials given to a platform location, i.e. savings in time, or distance travelled, to reach the platform between those

trials. Most studies of one-trial place memory in this task have not detected significant changes in escape latency savings or path length savings when the intervals between acquisition and retention trials were varied from 15s to as much as 6 hours (Morris et al., 1990; Steele and Morris, 1999; von Linstow Roloff et al., 2002; O'Carroll et al., 2006) (but see de Hoz et al., 2005). These studies suggest that memory strength is unaltered over that time period; however, the only systematic study of memory persistence in this task has provided a different account of memory decay over time. In this study Panakhova and colleagues (1984) investigated persistence of memory for a single acquisition trial up to 24h. Analysis of escape latencies during the retention trial showed that memory deteriorated rapidly with increasing retention intervals. Rats took more time to find the platform when the retention interval was extended from 1min to 1h and progressive decline was observed with 6 and 24h. Still, retention trial latencies were significantly different from acquisition trial latencies after 24h (Panakhova et al., 1984). In a related study, Bolding and Rudy (2006) investigated retention of memory for 10 consecutive trials (2min ITI) after 10min, 30min, 4h, 24h and 48h. Assessment of performance as selective search during probe trials, in which the escape platform was only made available after 20s, provided mixed results depending on the used measure of performance. The conventional quadrant measure, which compares the time spent in the target quadrant to the average of time spent in three other quadrants, revealed significant preference for the target quadrant up to 4h (and apparently no significant difference between performance at 4 and 24h). However, the comparison of the time that rats spent in the target quadrant with the time that they spent in their second most preferred quadrant revealed much faster decay of memory, with significant preference of the target quadrant observed only up to 30min (Bolding and Rudy, 2006). This study clearly reveals how different measures of performance can provide different readings of memory strength and how important it is to choose appropriate measures of behaviour. Overall, the use of different measures of performance in these studies makes it difficult to compare different accounts of allocentric place memory persistence in the watermaze

DMP task, as well as to assess the impact of different behavioural factors, such as training procedures and others, on memory decay between studies.

One factor that has been proposed to determine the persistence of rapidly acquired allocentric place memory is the nature of memory motivation. Given the contrasting levels of memory longevity observed in the radial-arm maze task (maximum reported of 6h; Maki et al., 1984) and in the DMP task in the watermaze (24h; Panakhova et al., 1984), Bolhuis and colleagues (1985) decided to investigate the possibility that the appetitive and aversive nature of these tasks contributed to the different observed rates of memory decay [2]. For this purpose they compared persistence of memory in the traditional appetitively-motivated radial-arm maze task and in a new aversively-motivated radial water maze task (Bolhuis et al., 1985; Buresova et al., 1985b). Tasks and protocols were similar, the difference being that in the new radial water maze task animals had to swim to a submerged platform made available at the end of each arm. Animals were allowed to stay in the platform for 20s, after this time the platform was collapsed and the animals had to return to the central platform. The central platform would be lowered 15s later forcing the animal to swim again. Both mazes had 8 arms and a variable retention interval was interposed between choices 4 and 5. Exponential decay of memory was observed in both tasks, but faster forgetting was observed in the traditional version of the radial-arm maze.

In this thesis two new behavioural tasks are developed to study rapidly acquired allocentric place memory. *First*, a new food-reinforced DMP task is developed which allows the separate study of encoding and retrieval of one-trial allocentric place memory in an open field "*event-arena*" (EXPERIMENTS 1-2). Performance in this task is shown to require visuo-spatial information and not to rely on odour or idiothetic cues (EXPERIMENTS 3-4). *Second*, a new protocol for the DMP task in the watermaze is also developed (EXPERIMENTS 8-10). As referred above, traditional versions of the DMP task in the watermaze measured place learning as a

[2] Other possible explanations referred by the authors were that only one item had to be remembered in the watermaze task, as opposed to several in the radial-arm maze, and that both tasks required different strategies, namely *win-stay* in the watermaze, and *win-shift* in the radial-arm maze.

reduction in escape latencies or in path lengths across successive trials given to a platform location. However, escape latencies and path lengths display a variability that is strongly influenced by chance (e.g. when the rat "bumps" into the platform), and may be reduced efficiently through systematic search strategies and the use of single beacon cues (e.g. Morris, 1981; Buresova et al., 1985a; Schenk and Morris, 1985; Jacobs and Schenk, 2003b). Furthermore, escape latency and path length savings strongly depend on performance during encoding trials, which is determined by chance. In contrast, measures of search preference, such as quadrant analysis and zone analysis, have long been recognized as a reliable and sensitive measure of allocentric place memory in reference-memory versions of the watermaze task, in which the platform location remains constant across trials and testing days (e.g. Morris, 1981; Buresova et al., 1985a; Schenk and Morris, 1985; Moser et al., 1998). The new DMP protocol presented in this thesis introduces probe trials to include zone analysis as a measure of performance. In EXPERIMENTS 5 and 8-10, comparable measures of search preference are used in both the event-arena and the watermaze DMP tasks to determine and compare the persistence of both appetitively-motivated and aversively-motivated one-trial allocentric place memories.

1.3.2. Contribution of NMDA and AMPA receptor activation in the hippocampus to encoding and retrieval of one-trial place memory in the event arena (Expt. 6-7)

Many theoretical models emphasize the importance of the hippocampus for rapid encoding and subsequent retrieval of various types of "relational" memory, including allocentric place memory, that require representations of mutual relationships between multiple features (O'Keefe and Nadel, 1978; O'Reilly and Rudy, 2001; Morris et al., 2003; Eichenbaum, 2004; Matus-Amat et al., 2004; Nakazawa et al., 2004). According to these models, encoding of one-trial place memory requires the induction of hippocampal synaptic plasticity, mediated by NMDA receptors (Bliss and Collingridge, 1993; Martin et al., 2000b), to conjoin a feature configuration

defining a place into a memory representation. Retrieval requires fast excitatory transmission through hippocampal synapses, essentially mediated by AMPA receptors (Davies and Collingridge, 1989; Lambert and Jones, 1990), to activate the stored place representation after perceiving elements of the feature configuration. Alternatively, it has been suggested that the neural representation of trial-specific places in familiar environments over minutes to hours may not require hippocampal NMDA receptor activation (Shapiro and O'Connor, 1992; Caramanos and Shapiro, 1994; Kesner and Rolls, 2001). EXPERIMENTS 6-7 examined the contributions of hippocampal NMDA and AMPA receptor activation to encoding and retrieval of one-trial place memory in the newly developed one-trial place memory task in the event arena. EXPERIMENT 6 examined the effects of intra-hippocampal infusions of AP-5 or CNQX, competitive antagonists at the NMDA and AMPA receptor, respectively (Watkins et al., 1990), on encoding and retrieval of one-trial place memory. In EXPERIMENT 7, electrophysiological measurements were conducted to establish that, if infused exactly as in the behavioural experiment, AP-5 selectively blocks the induction of synaptic plasticity and CNQX reduces fast excitatory synaptic transmission in the hippocampus *in vivo*.

1.3.3. Characterizing "weak" and "strong" memory encoding events in the watermaze (Expt. 11-14)

Both the amount and pattern of training have long been established to influence long-term retention of memory across learning paradigms in a variety of species (e.g. Carew et al., 1972; Roberts and Kraemer, 1982; Tully et al., 1994; Menzel and Muller, 1996; Hermitte et al., 1999; Muzzio et al., 1999), including rodents (e.g. Glickman, 1961; Deutsch, 1962; McGaugh, 1966; Goodrick, 1973; Mitchell, 1973; Domjan, 1980; Roberts and Dale, 1981; Fanselow and Tighe, 1988; Kogan et al., 1997; Josselyn et al., 2001; Genoux et al., 2002; Scharf et al., 2002). In the watermaze, evidence supporting a facilitative effect of spaced training on long-term retention of

memory has come from studies focusing on memory acquired over several days of training. Spacing of acquisition trials over days was firstly shown to improve long-term retention of memory for a new platform location in an eight-trial reversal task (Morris and Doyle, 1985). In another study, genetically induced deficits in long-term reference memory were rescued by spacing trials within days, over several days of training (Kogan et al., 1997). An improvement of long-term reference memory has also been shown in naïve rats when trial blocks were spaced over a day or several days of training (Spreng et al., 2002; Bolding and Rudy, 2006; Sisti et al., 2007). EXPERIMENTS 11-13 investigated if both the number and the temporal distribution of acquisition trials modulated memory strength for a single training session in the DMP task. Intervals of 5-10 min have been shown to facilitate the formation of long-term memory by multi-trial encoding (e.g. Fanselow and Tighe, 1988; Kogan et al., 1997; Josselyn et al., 2001; Genoux et al., 2002) and the induction of long-lasting synaptic plasticity phenomena *in vitro* by repeated synaptic stimulation (e.g. Reymann et al., 1985; Frey et al., 1993; Scharf et al., 2002). These experiments compared *i)* memory for one, three or six acquisition trials after retention intervals of 6h or 24h and *ii)* "massed" training comprising inter-trial intervals (ITIs) of 15s to "spaced" training comprising ITIs of 10min. Some watermaze studies suggest that the period of time that rats spend on the escape platform can be used to acquire information on the relative position of escape to environmental cues (Sutherland and Linggard, 1982; Keith and McVety, 1988; Whishaw, 1991). Based on these studies, a condition in EXPERIMENT 11 further investigated if the time rats were allowed on the platform (6 or 30s) would determine retention of 6h memory for a single acquisition trial. Finally, studies of "latent learning" in the watermaze have shown that animals do not have to swim to the platform to learn its location. After a period of pretraining with standard "swim" trials, memory for a new platform location can also be acquired after "placement" trials in which the rat is simply placed on the platform for a brief period of time (e.g. 30s; see Whishaw, 1991). Placement trials, however, are thought to produce weaker memory than standard swim trials (Whishaw, 1991). EXPERIMENT 14 established 3 spaced (10min ITI) standard trials as strong encoding events in the watermaze, producing strong

long-lasting memory detectable 24h after training, and 3 spaced (10min ITI) placement trials as weak encoding events, producing weak memory detectable 30min, but not 24h, after training.

1.3.4. Investigating synergistic interactions between "weak" and "strong" encoding events in the watermaze (Expt. 15-17)

In EXPERIMENTS 15-17, weak and strong encoding events were combined, similarly to "weak-before-strong" and "strong-before weak" STC electrophysiological paradigms characterized in rat hippocampal slices (Frey and Morris, 1997, 1998b), to investigate if STC mechanisms could be detected during the formation of allocentric place memory in the watermaze. It was predicted that memory for the weak encoding event would be stabilized into long-lasting memory when its encoding event was followed, or preceded, by a strong encoding event leading to the formation of long-lasting memory. Each encoding event occurred in a different watermaze room, with different geometry and configuration of available extra-maze cues, to attenuate proactive or retroactive interference between generated memories (Underwood, 1957).

1.3.5. Investigating a behavioural analogue of the "strong-before-strong" paradigm in the watermaze (Expt. 18-21)

Dependence of long-term memory for "strong" encoding events on protein synthesis in the hippocampus (Expt. 18-19)

Formation of long-term reference memory in the watermaze, which is acquired over several days of training to a fixed platform position, has been shown to depend on both transcriptional and translational mechanisms in the hippocampus (e.g. Guzowski and McGaugh, 1997; Kogan et al., 1997; Meiri and Rosenblum, 1998; Guzowski et al., 2000; Guzowski et al., 2001; Gusev et al., 2005; Plath et al., 2006; McGauran et al., 2008). EXPERIMENT 18 established that long-

term memory for a few encoding trials comprising a short training session in the DMP task also depends on translational mechanisms in the hippocampus. In this experiment, bilateral intra-hippocampal infusions of the broad-spectrum protein synthesis inhibitor anisomycin 30min before strong encoding events in the watermaze were shown to disrupt the formation of long-term memory for the location of the platform.

Anisomycin is a bacterial antibiotic that produces translational arrest by inhibiting peptidyl transferase activity, and therefore peptide bond formation, in the ribosome (Grollman, 1967). The effect of both systemic and central administration of anisomycin on protein synthesis has been well characterized across species, brain structures and experimental conditions (Flood et al., 1973; Davis et al., 1980; Patterson et al., 1989; Rosenblum et al., 1993; Meiri and Rosenblum, 1998; Maren et al., 2003; Ben Mamou et al., 2006; Morris et al., 2006; Helmstetter et al., 2008; Wanisch and Wotjak, 2008). One obvious outcome of these studies, as well as of others comprising the use of different broad spectrum protein synthesis inhibitors (e.g. Milekic et al., 2006), is that the magnitude, diffusion and time course of protein synthesis inhibition varies with the route and dosage of drug administration and targeted brain regions. In view of this, the rigorous assessment and interpretation of PSI-induced behavioural effects depends on the equally careful characterization of protein synthesis inhibition produced in each experimental setting. This becomes even more relevant when the extent and persistence of amnesia itself seem to depend on the magnitude and duration of protein synthesis inhibition (Flood et al., 1973; Flood et al., 1975; Barraco and Stettner, 1976; Davis and Rosenzweig, 1978; Milekic et al., 2006). In EXPERIMENT 19, autoradiographic imaging and quantitative densitometric analysis of [^{14}C] L-leucine uptake into the brain (Smith, 1991) were used to assess the extent and duration of protein synthesis inhibition produced by bilateral intra-hippocampal infusions of anisomycin in behavioural experiments.

Investigating a behavioural analogue of the "strong-before-strong" paradigm in the watermaze (Expt. 20)

After establishing the time course and local specificity of protein synthesis inhibition produced by intra-hippocampal infusions of anisomycin (Expt. 19), and the dependence of long-term memory for strong encoding events on protein synthesis in the dorsal hippocampus (Expt. 18), EXPERIMENT 20 investigated if long-term memory for a strong encoding event occurring during anisomycin-induced protein synthesis inhibition in the dorsal hippocampus could be rescued if that encoding event was preceded, one hour apart, by a second strong encoding event producing long-term memory. This behavioural protocol was designed to mimic the "strong-before-strong" STC paradigm characterized in electrophysiological experiments (Frey and Morris, 1997).

Investigating overlap of neuronal ensembles recruited by two distinct encoding events in the watermaze (Expt. 21)

STC-like synergistic interactions between two strong encoding events can only be observed if *i)* those events induce up-regulation of protein synthesis, and if *ii)* both events recruit independent, but overlapping, populations of neurons. A relatively new and exciting cellular imaging method allows assessing the overlap of neuronal ensembles recruited in the hippocampus by two discrete behavioural events. This method, *Arc/Homer1a* catFISH (cellular compartmental analysis of temporal activity by fluorescent *in situ* hybridization), capitalizes on the different structure of two effector immediate-early genes (IEG; effector IEGs can directly modulate specific cellular functions), *Arc* and *Homer1a* (*H1a*), and the precise onset and shut-off of synaptic activity-driven immediate-early gene transcription in neurons (Vazdarjanova et al., 2002; Guzowski et al., 2005). Transcription of *Arc* and *H1a* is tightly regulated by activity and rapidly induced in the hippocampus and neocortex following spatial exploration (Guzowski et al., 1999; Vazdarjanova et al., 2002; Ramirez-Amaya et al., 2005). *Arc* mRNA is derived from a short primary transcript whereas *H1a* mRNA is generated from a longer and more

complex primary transcript (Bottai et al., 2002). For this reason, *Arc* intra-nuclear transcription foci (INF) can only be detected from the period ~2-15min from induction, whereas *H1a* INF can only be detected ~25-40min later (Vazdarjanova et al., 2002). In view of this, by using double label fluorescence *in situ* hybridization with an *H1a* 3'-untranslated region (UTR)-specific riboprobe and a full length *Arc* riboprobe it is possible to identify neurons that were activated at two different time windows previous to sacrifice. Neurons activated ~25-40min before sacrifice (Epoch 1) will express *H1a* INF (*H1a*$^{+}$ *cells*) and neurons activated ~2-15min before sacrifice (Epoch 2) will express *Arc* INF (*H1a*$^{+}$ cells). Importantly, neurons expressing both *H1a* and *Arc* INF (*H1a*$^{+}$*/Arc*$^{+}$ cells) would have been activated in both epochs. In one study *H1a/Arc* catFISH was used to investigate the overlap of neuronal ensembles recruited in the hippocampus by two distinct behavioural events (Vazdarjanova and Guzowski, 2004). In this study rats were exposed to one environment for 6min (Epoch 1), returned to their home cage for 20min, and then exposed for other 6min to the same environment, or to a novel environment, before sacrifice (Epoch 2). Exposure to the first environment resulted in the expression of *H1a* INF in ~30-40% of CA1 cells in both conditions (including double-labelled *H1a*$^{+}$*/Arc*$^{+}$ cells). Exposure to the second environment resulted in the expression of *Arc* INF in ~30-35% of CA1 cells (also including *H1a*$^{+}$*/Arc*$^{+}$ cells). When animals were exposed to the same environment twice, both *H1a* and *Arc* INF were observed simultaneously in ~30% of CA1 hippocampal cells, suggesting that the same population of neurons was activated in both epochs. When rats were exposed to different environments overlap was reduced to ~15-20% of cells, suggesting that two distinct, but overlapping, populations of neurons were activated during both events. The first objective of EXPERIMENT 21 was to use the *H1a/Arc* catFISH technique to determine if two strong encoding events, occurring in different watermaze rooms, would recruit an overlapping population of neurons in the rat hippocampus, and specifically in the CA1 region.

Investigating up-regulation of mRNA and protein synthesis following strong encoding events in the watermaze (Expt. 21)

By using *Arc/Homer1a* catFISH in EXPERIMENT 21 it was also possible to investigate if strong encoding events in the watermaze would up-regulate the synthesis of two important mRNA species that have been associated with the consolidation of hippocampal synaptic plasticity and hippocampal-dependent memories (see Arc studies below; for Homer 1a: Brakeman et al., 1997; Kato et al., 1997; French et al., 2001; Igaz et al., 2004; Ronesi and Huber, 2008; Inoue et al., 2009).

From the IEGs investigated in the field of learning and memory, the effector IEG *Arc* (activity-regulated cytoskeleton-associated protein; Lyford et al., 1995), also known as *Arg3.1* (Link et al., 1995), has probably been the one receiving the most attention. This has been due not only to its tight activity-dependent regulation, which allows mapping behaviourally defined neuronal networks (Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004; reviewed by Guzowski et al., 2005; Vazdarjanova et al., 2006; Kubik et al., 2007; Miyashita et al., 2008), but also to several other reasons. *First*, *Arc* mRNA, which is present at very low basal levels in resting hippocampal neurons, is strongly up-regulated after induction of LTP in the hippocampus *in vivo* (French et al., 2001; Waltereit et al., 2001; Miyashita et al., 2009) or following experiences that involve hippocampal-dependent learning (Guzowski et al., 2001; Gusev et al., 2005; Miyashita et al., 2009). *Second*, induction of *Arc* mRNA *in vivo* is NMDA-dependent (Link et al., 1995). *Third*, NMDA-receptor dependent targeting of *Arc* mRNA to dendrites close to recently activated synapses has been shown after induction of LTP in the hippocampus (Steward et al., 1998; Steward and Worley, 2001; Moga et al., 2004; Huang et al., 2007). *Fourth*, induction of LTP in the hippocampus also results in the accumulation of Arc protein in dendrites and on its enrichment at sites of local synaptic activation, suggesting that Arc protein is synthesised locally (Steward et al., 1998; Moga et al., 2004; Rodriguez et al., 2005). *Fifth*, spatial exploration induces Arc protein expression in the

hippocampus (Ramirez-Amaya et al., 2005). *Finally*, studies using Arc antisense oligodeoxynucleotides (ODN) have shown that translation of Arc protein is required for the maintenance of late-phase synaptic plasticity in the hippocampus (Guzowski et al., 2000; Messaoudi et al., 2007; Waung et al., 2008) and the formation of long-lasting hippocampal-dependent memories (Guzowski et al., 2000; McIntyre et al., 2005). Severe deficits in late-phase LTP in the hippocampus and in hippocampal-dependent long-term memory have been also observed in Arc null knockout mice (Plath et al., 2006). Given the requirement for Arc protein translation on both LTP and memory consolidation (recently reviewed by Tzingounis and Nicoll, 2006; Bramham, 2008; Bramham et al., 2008; Miyashita et al., 2008), and the requirement for protein synthesis in STC mechanisms, immunohistochemical analysis of tissue obtained in Experiment 21 was also used to determine if strong encoding events in the watermaze would induce Arc protein expression in the rat CA1 hippocampal region.

Chapter 2: Materials and methods

2.1. Subjects

Experiments 1-6: Behavioural studies in the "event" arena

Thirty-one adult male Lister Hooded rats (Charles River, Margate, UK) were used for Expt. 1 and 6 (n=15) and Expts. 2-5 (n=16). They were single housed in a temperature (20–23°C) and humidity (40%–55%) controlled animal room with an artificial light/dark cycle (lights on 7:00 A.M. to 7:00 P.M.). The rats had *ad libitum* access to water and were fed a restricted diet (18–24g per day of standard rat chow, RM1; Special Diet Services, Witham, Essex, UK) to maintain them at ~85% of their free-feeding weight estimated according to a previously established growth curve. They weighed 200–250g and were 8–10 weeks old at the beginning of the food restriction. After completion of Expt. 1, the same batch of animals (n=15; ~6.5 months of age) was tested in a behavioural pharmacological study (Expt. 6). This study began with bilateral implantation of infusion guide cannulae and one rat was excluded after surgery because of bad health, leaving n=14. The rats were kept on a moderate food-deprivation schedule and housed as described above, but had *ad libitum* access to food from day 1 before until day 2 after surgery.

Experiment 7: Electrophysiology

Thirty-nine naive male Lister Hooded rats (~250–390g) with *ad libitum* access to food, but otherwise housed as the rats in the previous experiments, were used.

Experiments 8-18 and 20: Behavioural studies in the watermaze

One hundred and two adult male Lister Hooded rats were used in Expts. 8-10 (n=20), 11 (n=18), 12-14 (n=16), 15-17 (n=20), 18 (n=12) and 20 (n=16), respectively. One of the rats of Expt. 20 was excluded due to bad health after surgery, leaving n=15. The rats used in Expts. 8-17 weighed ~220-280g at the start of the experiments and were housed 2 *per* cage in the animal room described above. The rats used in Expt. 18 and 20 weighed ~230-300g during cannulae implantation and ~330-410g at the start of the behavioural training, a week later. As experiments 18-21 were run in a new laboratory, the animals were housed (4 *per* cage) in a new animal room; temperature, humidity and light conditions were kept constant between animal rooms. Animals used in experiments 8-21 had *ad libitum* access to food and water.

Experiments 19 and 21: [^{14}C] L-leucine autoradiography, fluorescence in situ hybridization and immunohistochemistry studies

Thirty-eight adult male Lister Hooded rats were used in experiments 19 (n=18) and 21 (n=20). Rats used in Expt. 19 weighed ~240-320g during canullae implantation and ~350-420g when the radiolabelled leucine injections took place, a week later. Rats used in Expt. 21 weighed ~230-280g at the start of the behavioural training and ~360-420g when sacrificed, ~1.5 weeks later.

All experiments described in this thesis were conducted during the light phase of the light/dark cycle. Rats were always habituated to handling by the experimenter before the start of experiments (at least 5 days; approx. 2min *per* animal each day). The Principles of Laboratory Animal Care (National Institutes of Health publication number 86-23, revised 1985) and Home Office regulations for animal experimentation were followed.

2.2. Apparatus

2.2.1. The "event arena"

The arena (1.6x1.6m) had a 7x7 grid of 49 circular holes covered by plastic lids; it stood 75cm above the floor and it was bounded by four walls of clear Perspex (30cm high). In the centre of each wall there was a rectangle shaped entrance with a sliding door and a start box (25x25x25cm) made from clear Perspex behind it (see Fig.2.1a-b). The arena surface was covered by a thin layer of sawdust and the second and sixth holes in row 4 were each covered by a landmark, a stack of golf balls and a pyramid (Fig.2.1b). Sandwells (6cm diameter, 3.5 cm deep) could be put in the remaining holes, with their edges plane with the arena surface. The sandwells were filled with bird sand (Trilcot, Lincs, UK) up to 0.5–1cm below the edge (Fig.2.3.1c). One-half pellets (500mg) of rodent food (Bio-Serve, Frenchtown, NJ) were used as food reward. The sand was adulterated with thoroughly ground food pellets (23g per 2.5kg of sand), to reduce the possibility of rats using food odour to retrieve reward.

The arena was placed in a rectangular test room (2.6x4m) with white walls. The room comprised a "holding area," where rats could be kept in their home cages before and after trials and during the retention intervals. The holding area was separated from the rest of the room by a gray divider wall (see Fig.2.1b). Three-dimensional cues (star-shaped, ball-shaped, and cubic

lampshades of different colours) were mounted in this wall and in the room walls. One long wall was decorated with a pattern of big black rectangles and white stripes, and one short wall had a poster with black spots on a white ground on it. Normally, the test room was illuminated (100 lux) by wall-mounted halogen lamps. For tests in darkness (i.e., excluding light frequencies within the rats' visual spectrum; Expt. 4), an infrared light source (VISO10IR; Voltek, Stafford, Staffordshire, UK) mounted at the ceiling close to the infrared-sensitive camera provided the only illumination, and the experimenter wore infrared-sensitive night-vision goggles (Cobra Optics, Henfield, West Sussex, UK). The room was kept at ~22°C by ceiling ventilation.

The test room could be accessed via two gray doors in the two short walls. One door led to the corridor via which the rats were brought into the holding area. The other door gave access to a control room with a personal computer and a video recorder, both of which were connected to a wide-angle video camera mounted at the ceiling above the arena. The personal computer ran dedicated LabView (National Instruments, Austin, TX) software, developed in the laboratory, to monitor trials and to take time measurements, in particular the dig time at different sandwells (see below, Measures of one-trial place memory), and for remote control of start-box doors. The video recorder was used to tape the trials.

2.2.2. The watermaze

The watermazes were 2m diameter and contained water at 25±1ºC made opaque by the addition of 200ml of latex liquid (Cementone-Beaver Ltd, Buckingham, UK). Water was changed daily using automatic filling and draining systems. A 12cm diameter "Atlantis platform", a polystyrene platform that becomes available by rising from the bottom of the pool (Spooner et al., 1994), was submerged ~1.5cm below the water surface when raised so as to be

hidden from view at the water surface. The rat's behaviour was monitored by an overhead video camera connected to a video recorder and an on-line data acquisition system located in an adjacent room. The data acquisition system digitized the path taken by the animal and computed spatial parameters (e.g. escape latencies and time in zone).

Experiments 8-17

These experiments used two different watermazes, located in different rooms on different floors of the laboratory. The watermaze rooms differed in geometry and in the type and spatial configuration of visual cues available (see Fig. 2.2). Experiments 8-14 used only the watermaze located *upstairs*, whereas experiments 15-17 used both *upstairs* and *downstairs* watermazes, simultaneously.

Experiments 18, 20 and 21

Two new watermazes, located in new laboratory facilities, were used in these experiments. The new apparatus were placed in two different rooms with identical geometry but different cue configurations (see Fig. 2.3). These two rooms were also located on different floors of the new laboratory; experiment 18 used only the watermaze located *upstairs* and experiments 20 and 21 used both *upstairs* and *downstairs* watermazes, simultaneously. Adjacent to the watermaze rooms were control/holding rooms where animals were held between training trials and from which the experimenter monitored the behaviour. Watermaze and control/holding rooms were separated by curtains.

Figure 2.1: “Event arena” apparatus

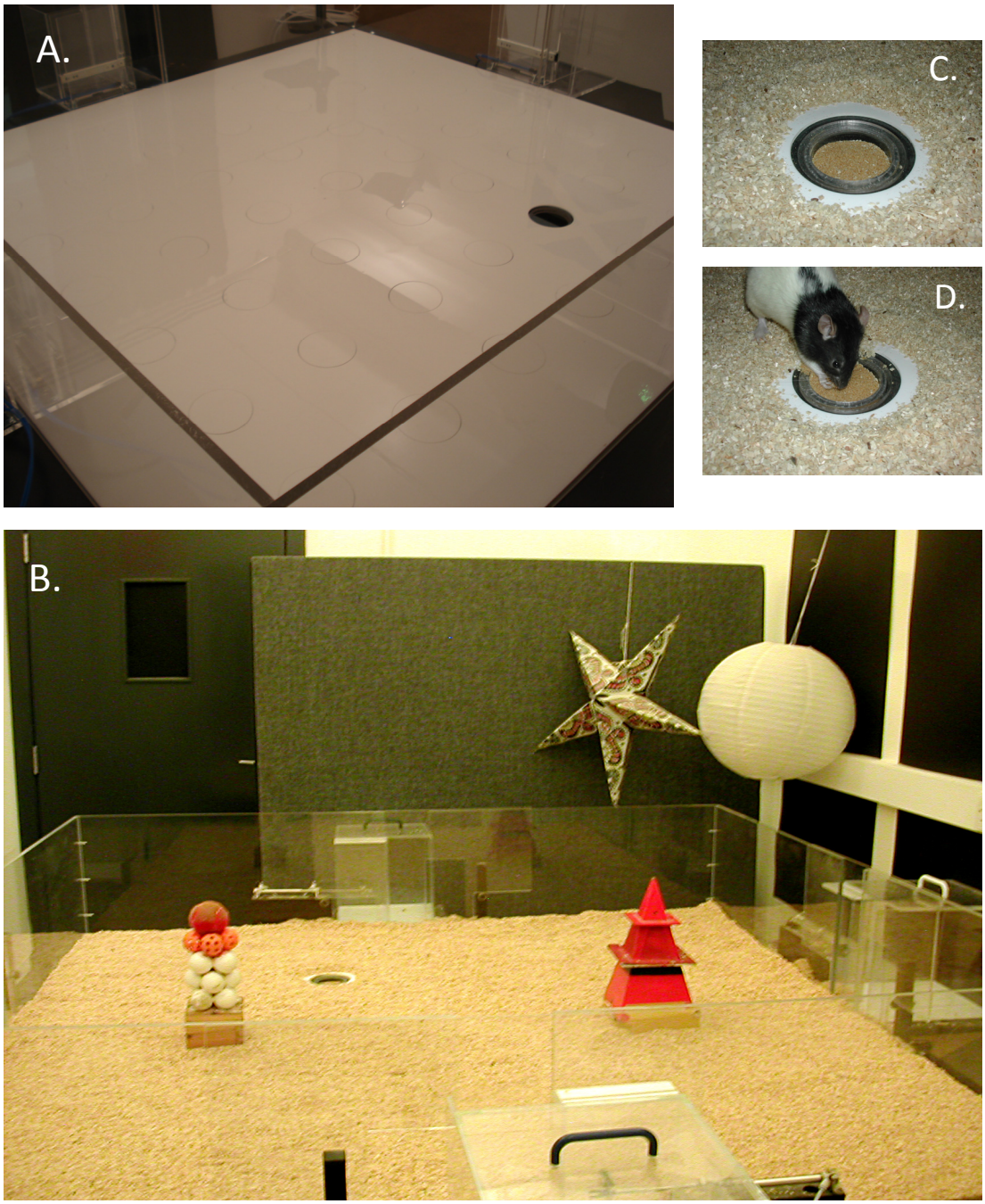
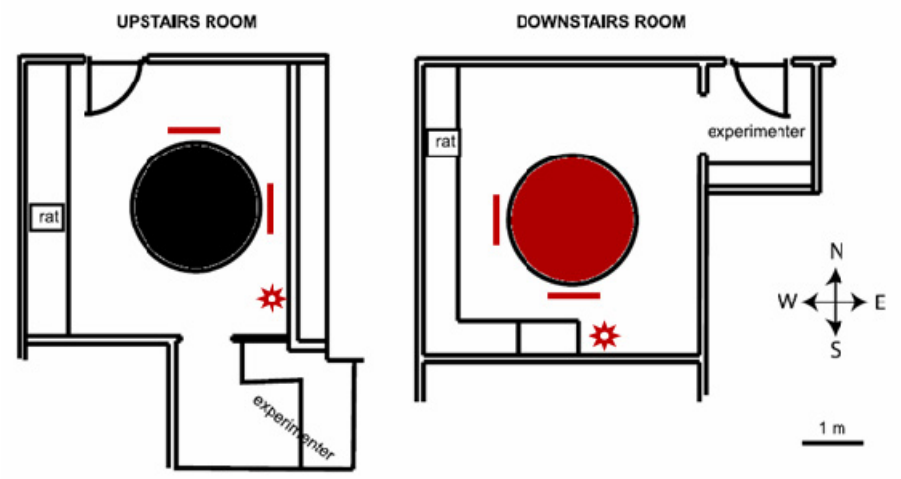


Figure 2.1. *The “event” arena apparatus.* ***A)*** The arena floor has a 7x7 grid of circular holes covered by lids that could be removed to insert sandwells. ***B)*** The surface of the arena was covered by sawdust. The arena contained two intra-maze cues, a stack of golf balls and a pyramid. Bi-dimensional and tri-dimensional extra-maze cues were mounted on the surrounding walls. ***C)*** The sandwells are filled up to 0.5-1cm below the edge with bird sand and ground food (to reduce the possibility of the rats using food odour for guidance). ***D)*** Dig time was used as a measure of performance. The rat was considered digging when it was observed putting the front paws on or into the sand with the snout directed downward to the sand (see Measures of one-trial place memory).

Figure 2.2: Watermaze apparatus (Expt. 8-17)

A. Diagram of the upstairs and downstairs watermaze rooms



B. Upstairs watermaze

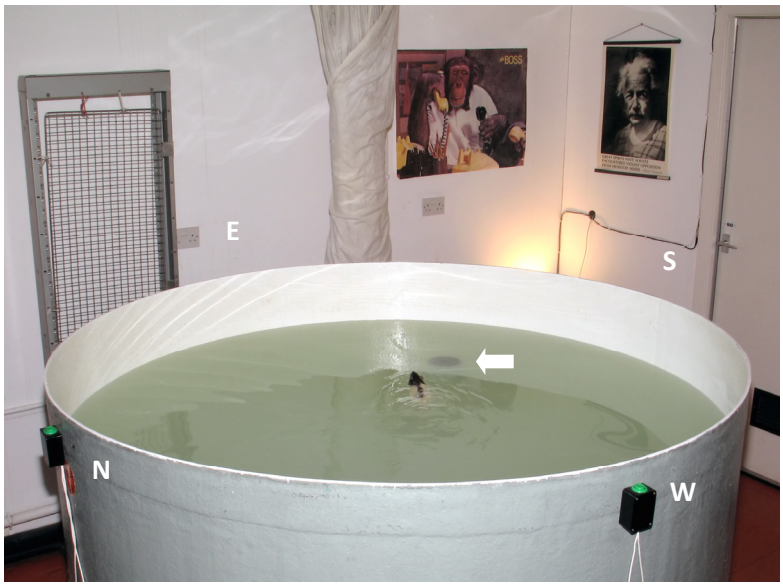


Figure 2.2. *Watermaze apparatus (Expts. 8-17).* ***A)*** Diagram of the upstairs and downstairs watermaze rooms [*adapted from* (de Hoz et al., 2003)]. The 2 different watermazes used in experiments 8-17 were located in different rooms in different floors of the laboratory. The upstairs (left; black watermaze) and the downstairs (right; red watermaze) rooms differed in geometry and in configuration of extra-pool visual cues available. Metal racks (red bars) and rolled-up white curtains (red stars), both prominent cues, were present in both rooms but were positioned differently in each room. Holding areas for rat cages were located west of the mazes. The experimenter monitored the behaviour from "control" rooms located south (upstairs) or east (downstairs) of the testing rooms. ***B)*** Upstairs watermaze (downstairs watermaze not shown). The mazes were located in well-lit rooms with different configurations of extra-pool visual cues (e.g. posters on the walls); no cues were located within the pools. The rats had to escape to a platform (Atlantis platform) submerged approximately 1.5cm below the water surface platform (see B; white arrow). Trials began at north, west, south or east positions (N, W, S, E; white inset letters), with the rats facing the side walls.

Figure 2.3: Watermaze apparatus (Expt. 18, 20 and 21)

A. Upstairs watermaze

B. Downstairs watermaze

Figure 2.3. *The watermaze apparatus (Expts. 18, 20 and 21).* Watermazes used in experiments 18, 20 and 21. The different watermaze rooms had the same dimension and geometry but different configurations of cues. Control/holding rooms were located south of the mazes (black S). Experiment 18 only used the watermaze located upstairs; experiments 20 and 21 used both upstairs and downstairs watermazes.

2.3. New delayed matching-to-place tasks in the “event arena” and the watermaze

2.3.1. The one-trial place memory task in the “event arena”

The new task was designed to enable the study of the neural substrates of encoding and retrieval of one-trial allocentric place memory. This required robust measures of place memory for discrete trials and a separation of encoding and retrieval phases by several minutes to allow, for example, intra-cerebral drug infusions and appropriate drug diffusion between the phases.

Shaping and habituation

All food restricted animals were shaped to dig for food in sandwells placed in their home cages during the first week of food restriction. In the second week, they were habituated to the test environment and trained to search and dig for food in the arena. On the first day of habituation, the rats were put into the arena for 5min with no sandwell in place and all holes closed. For days 2–6, they received one daily habituation trial. In habituation trials, the rat was put in a start box and, after 20s, allowed access to the arena. The rats started from a different start box each day, so that they had started from all four boxes at least once by the end of the habituation period. In the first habituation trial, one sandwell with one-half of a pellet of food buried near the surface was placed in the centre location of the arena. In the following habituation trials, one sandwell with the one-half pellet deeply buried on the bottom was in the location immediately in front of the start box opposite to the one from which the rat started the trial. At the end of the 5 day habituation period, all rats readily searched and dug for food in the arena, so that a complete habituation trial would typically take 1.5–5min (including the 20s in the start box and the 60s allowed to eat the food).

One-trial place memory protocol

The protocol comprised one trial per day consisting of two phases: *i)* an encoding phase, in which the rats had to search the arena to find food in a trial-specific place and to encode this place in memory; and *ii)* after a retention interval, a retrieval phase, in which the rats could use one-trial place memory to find food in the same place as in the preceding encoding phase (Fig. 2.4a). A key aspect of the task is the use of different places across days to enable the successive examination of one-trial encoding and retrieval across a range of conditions in within subjects designs.

Trials were always separated by at least one day, with five to seven trials per week. The trials started with the encoding phase: the rat was put in a start box and after 20s was allowed access to the arena. Once the animal had entered the arena, the access door was closed preventing re-entry of the animal to the start box. In the encoding phase, the rat had to search for an open sandwell in one particular location and to dig to retrieve the buried food reward. All other locations were closed and covered with sawdust. After the rat had retrieved the food, it was allowed 60s to eat the reward and then placed in its cage in the holding area for the duration of the retention interval. The retrieval phase started with the rat being put into a different start box from the one used at the beginning of the encoding phase [to encourage allocentric orientation based on relationships among multiple visual cues (Eichenbaum et al., 1990)] for 30s until the door was opened. In the retrieval phase, the rat could find food in a sandwell in the same location as in the encoding phase (the "correct" location), but four additional sandwells, without reward, were open in four "novel" locations. The rat was returned to its cage 60s after it had retrieved the food. During standard trials, the food reward in the retrieval phase was buried in the correct sandwell as in the encoding phase. During probe trials, none of the five sandwells in the retrieval phase contained a reward, and the rat was left searching and digging in the sandwells for a total of 60s (counted from the moment it had completely left the start box). After 60s, the experimenter would enter the room and would place a reward on the surface of the

correct sandwell, so that the rat could retrieve it. The purpose of probe trials was to obtain a "dig-time" measure (see below) and to render the use of olfactory cues emanating from the food reward impossible.

Correct and novel locations were changed daily. Locations in a trial were never directly adjacent, and they were chosen so that the different quadrants, as well as central and peripheral regions of the arena, were equally associated with correct or novel locations over days. The four locations forming a triangle in front of each start box were never used (except for habituation), because of the high probability that the rat would dig in these locations just by chance when exiting the start box. Because pilot tests suggested that the proximity of the sandwells to the rat's start box influences the first choice, all four start boxes were used in a counterbalanced way. To minimize the possibility that rats could use odour traces to find food, the sand used in the different locations was mixed between phases and trials, and the sawdust was whirled between phases and rats.

Measures of one-trial place memory

Measures of one-trial place memory taken during the retrieval phase were *i)* the rat's first choice (i.e., in which sandwell it dug first), *ii)* errors (i.e., the total number of sandwells in novel locations in which the rat dug before digging in the sandwell in the correct location), and *iii)* the dig time at correct and novel sandwells during the 60s retrieval phase in probe trials. "Digging" was defined as the rat putting both front paws on or into the sand with the snout directed downward to the sand (see Fig.2.1d). In addition to scoring digging based on the video image, the sandwells were checked for traces of digging: a bump in the sand or sand spread around the sandwell reflected that the animal had dug at a given location. To normalize dig-time measures, a rat's dig time at a sandwell was expressed as a percentage of the overall dig time during the 60s retrieval phase of the probe trial (percentage of dig time at sandwell). The values expected

based on chance were 20% of the first choices and of the total dig time at each sandwell and an average number of two errors (Healy and Krebs, 1992; Clayton and Krebs, 1994).

2.3.2. The modified delayed matching-to-place task in the watermaze

Pretraining days

Pretraining days followed the original protocol described for the delayed matching-to-place task in the watermaze (Steele and Morris, 1999) (see Fig. 2.4b, *left*). Rats were given 4 *standard* trials a day to a platform position that varied between, but not within, days. Platform positions were distributed along inner (I) and outer (O) rings situated 40 and 70cm away from the centre of the pool (see Fig. 2.4b, *right*), respectively. Sequences of platform positions were designed so that all transitions between I-O, O-I, I-I and O-O rings would be used, and the establishment of a learning rule based on the inner or outer location of the platforms would be made difficult. Platform positions were never repeated within a sequence (including inter-probe and probe days; below). Trials began at north, west, south, or east, positions in a pseudorandom sequence, with the rats facing the walls. Rats were allowed a maximum of 2min to escape to the platform and 30s on the platform (available from the beginning of trials). If a rat failed to escape within 2min, the experimenter placed a hand above the platform in order to guide the animal. Between trials, the animals were placed in a cage under a heating lamp to dry and prevent hypothermia. Inter-trial intervals were varied between trials 1-2, but kept constant between trials 2-4.

Probe days

The new version of the DMP task in the watermaze introduced probe days in which retention trials were run as probe trials with the Atlantis platform made available only after 60s (see Fig. 2.4b, *left*). This enabled the assessment of performance for relevant experimental

conditions as the proportion of time that rats spent searching the zone where the platform was located during encoding trials. The *zone analysis* compared the time rats spent swimming in the correct platform zone (area defined by a 20 cm radius from the centre of the platform) to the time spent in 7 other equally sized zones (see Fig. 2.4b, *right*). The correct platform zone and the 7 additional zones were distributed symmetrically over the pool and were non-overlapping. The specific set of 8 platform positions analysed on a given probe trial was defined by the location of the platform during encoding. The zone analysis was calculated as follows: [(*time in correct zone*/*time in 8 zones*) x 100]. Probe trial latencies corresponded to the time rats took to intersect the area where the platform was located (crossing latencies).

In order to run within-subject designs rats were distributed into groups at the beginning of the experiments. Experimental conditions were counterbalanced for animal groups and probe days according to a Latin square-type design; platform positions, as well as start positions, were counterbalanced for conditions. In experiments comparing performance between one-trial and multi-trial encoding, daily start positions were further adjusted so that *i)* they were always kept constant for the retention trial and its preceding trial between conditions, and; *ii)* the starting position for the retention trial would be presented only once [e.g. 1 encoding trial (N-SEW), 3 encoding trials (EWN-S), 6 encoding trials (EWNEWN-S)]. A probe day series included as many probe days as conditions investigated in the experiment. Probe days were always preceded by standard pretraining days (inter-probe days); this was to minimise carry over effects between probe days (i.e. that the effects of different conditions on memory acquired on a probe day would affect learning on the subsequent probe day) and established the stability of performance within and between experiments. The inter-trial intervals between trials 1-4 were kept constant at ~15s during inter-probe days unless stated otherwise.

2.4. Experimental designs

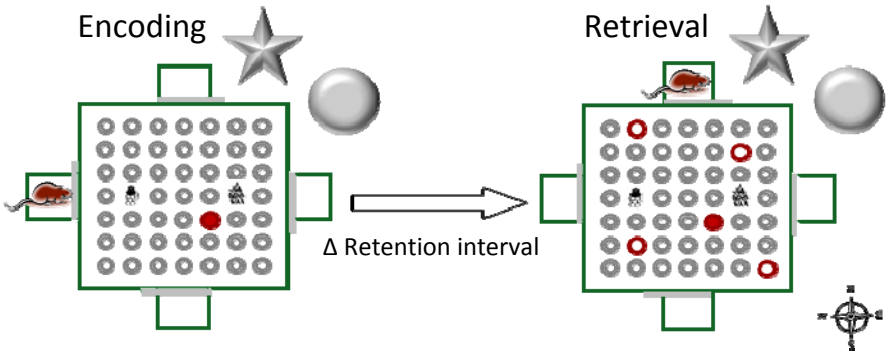
2.4.1. Event arena (within-subject designs)

Experiment 1: Acquisition of the task

Fifteen rats were used to develop the novel one-trial place memory protocol and to establish that performance would be sufficiently robust for the later drug infusion studies. After shaping and habituation as described above, these rats had been trained in a flavour–place paired-associate protocol (50 trials; 1 trial per day) (Day et al., 2003) as part of ongoing efforts in the laboratory to clarify factors influencing performance in different event-arena protocols. After this initial study, rats were given 18 trials (1 trial per day) with a 5min retention interval between the encoding and retrieval phases in the one-trial place memory task. Trials 1–16 and 18 were standard training trials; trial 17 was a probe trial. Before moving on to the surgery for the infusion studies of experiment 6, the 15 rats were first subjected to a few additional trials to ensure that, as required for the infusion studies, one-trial place memory persisted over a delay longer than 5min. For this purpose, performance was compared at a 5 and 45min retention interval in a counterbalanced within-subjects design. The rats were given 6 trials, with the retention interval alternating between 5 and 45min. Trials 3 and 6 were probe trials.

Figure 2.4: New one-trial place memory tasks in the "event arena" and the watermaze

A. One-trial place memory task in the event arena



B. Modified delayed matching-to-place (DMP) task in the watermaze

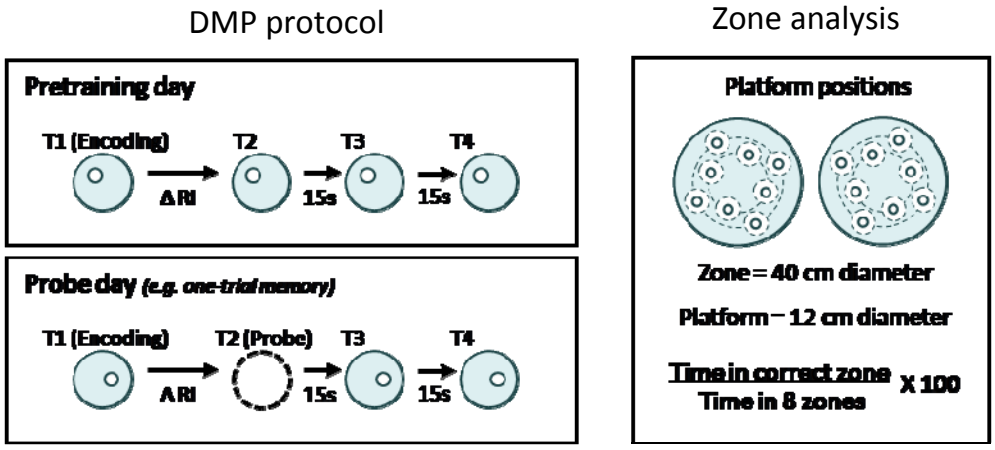


Figure 2.4. New *one-trial place memory tasks in the "event" arena and the watermaze.* ***A)*** A trial comprises an encoding phase and a retrieval phase. In the encoding phase, the rat must search for food reward buried in a sandwell (filled red circle) in a trial specific place; all other possible sandwell locations (open gray circles) are covered and covered by sawdust. In the retrieval phase, beginning after a variable retention interval during which the rat is returned to its home cage, food is buried in a sandwell in the same location as in the encoding phase (filled red circle), and sandwells without food are open in four novel places (open red circles); to find food efficiently in the retrieval phase, the rat must use one-trial place memory according to a win-stay rule. Start positions (North, South, West, and East) for encoding and retrieval phases are different promoting the use of allocentric place memory. ***B)*** *Left:* During pretraining days rats are given 4 *standard* trials (T1-T4), as previously described (e.g. Steele and Morris, 1999). The new protocol introduces probe days in which the retention trial (e.g. T2) is run as a probe trial (stippled gray circle). During a probe trial the escape platform (Atlantis platform) is only made available after 60s. This allows analyzing the percentage of time rats spend searching the zone where escape could be previously found (*zone analysis*). *Right:* The zone analysis compares the time rats spend swimming in the "correct" zone (a 20cm radius circular area centred on the platform location during encoding) to the time spent on 7 other equally sized zones, distributed symmetrically in the pool. Platform locations are distributed according to inner and outer rings (40 and 70cm away from the centre of the pool, respectively) to prevent overlapping of the zones analysed. Represented are two of 5 different sets of 8 platform positions used in the experiments RI-Retention interval

Experiments 2-5: Acquisition of the task and dependence of one-trial place memory on visuo-spatial cues and retention interval

Sixteen naive rats were used to replicate the initial task acquisition (Exp. 2) and to characterize factors underlying task performance, namely: effects of arena rotation between encoding and retrieval phases (Exp. 3); effects of darkness during retrieval (Exp. 4); and finally, dependence of one-trial place memory on retention interval (Exp. 5). Experiment 2 started immediately after shaping and habituation of the rats and comprised 16 training trials followed by one probe trial, with a 5min retention interval between encoding and retrieval phases. These 17 trials were run exactly as in experiment 1, using the same correct and novel sandwell locations. Experiment 5, followed experiment 2, and examined the effect of increasing retention intervals on one-trial place memory in the novel task. For this purpose, performance measures were compared in trials with retention intervals of 5, 60, 180, and 360min between encoding and retrieval phases in a counterbalanced within-subjects design. The experiment was run in two series; each series comprised four standard training trials with each retention interval, followed by pairs of alternating standard and probe trials at each of the four intervals. Experiment 3 examined whether the rats' performance may have relied on cryptic odour traces left on the arena surface. It was run in two series, with series 1 run before and series 2 run after the second series of experiment 5, allowing a direct comparison of the two experiments. Performance was tested with retention intervals of 20 and 360min, with the symmetric arena rotated by 90 or 180° between encoding and retrieval phases; the configuration of intra-arena landmarks and extra-arena landmarks was kept constant. If rats relied on cryptic odour cues on the arena surface, performance should be disrupted by arena rotation. Each series in experiment 3 comprised one pair of standard and probe trials for the 20 and the 360 min delay, with the order of the two retention intervals counterbalanced. Finally, experiment 4 examined the effects of darkness during retrieval to verify that normal task performance relied on visuo-spatial cues. To habituate the rats to sudden lighting changes, they were subjected to two standard training trials with a

5min retention interval during which the test room (including the holding area) was dark. After this habituation, probe trials (separated by an additional standard training trial with a 5min retention interval in darkness) were conducted under two different conditions in a counterbalanced within-subjects design. In one condition, the retrieval phase was conducted in light and in the second condition in darkness. In both conditions, the retention interval was 20min (because this was also used in the infusion studies) and it was dark during the retention interval (to control for a possibly startling effect of sudden lighting changes). Only one series of such probe trials was conducted, because repeated retrieval phases in darkness might have encouraged the rat to acquire non-visual strategies (such as orientation based on localized sound sources).

Experiment 6: Dependence of encoding and retrieval of one-trial place memory on activation of glutamate receptors in the hippocampus

The one-trial place memory task was run as described for Expts. 1-5. The retention interval between the encoding and retrieval phases and the time points for intra-hippocampal infusions were chosen to be identical to those used in previous studies of flavour–place memory (Day, et al. 2003): the retention interval was 20min, and infusions started 15min before the encoding phase or 15min before the retrieval phase. Infusions were conducted in the holding area of the experimental room. After recovery from surgery, the rats were subjected to four standard training trials (post-surgery habituation trials) to ascertain that one-trial place memory was still above chance and unaffected by mock infusions. Before the encoding or retrieval phase of the third and fourth trials, rats received mock infusions (eight rats before encoding of trial 3 and retrieval of trial 4 and seven rats vice versa). A mock infusion was identical to a real infusion, except that syringes and tubing were empty. The effects of hippocampal drug and ACSF infusions on encoding and retrieval of one-trial place memory were studied in a within-subjects design, with the order of the different infusion conditions counterbalanced between animals.

Infusion days were alternated with days without infusion. On infusion days, probe trials were conducted, whereas on the days without infusion, standard training trials were run. Experiment 6a examined the effects of hippocampal infusion of the NMDA receptor antagonist AP-5 before encoding and retrieval (four infusion conditions: AP-5 before encoding, AP-5 before retrieval, ACSF before encoding, ACSF before retrieval). As we shall see, this experiment revealed that NMDA receptor blockade disrupted encoding but not retrieval of memory. This implies that AMPA receptor blockade would also disrupt encoding, because NMDA receptor-mediated induction of synaptic plasticity requires AMPA receptor-mediated postsynaptic depolarization (Bliss and Collingridge, 1993). The interesting outstanding issue, however, was whether, in contrast to NMDA receptor blockade, AMPA receptor blockade would also impair retrieval, as predicted by the theoretical positions described above. Furthermore, limiting the number of infusion conditions could help to decrease the risk of problems associated with many intra-cerebral infusions (gliosis, infections, cannula blockade) while increasing the statistical power of the design. Thus, experiment 6b examined the effects of hippocampal infusion of the AMPA receptor antagonist CNQX only before retrieval (two infusion conditions: CNQX before retrieval, ACSF before retrieval).

2.4.2. Watermaze (within-subject designs)

Experiments 8-10: Persistence of one-trial place memory

Persistence of one-trial place memory in the DMP task in the watermaze was investigated in three different experiments using a single batch of animals. Strength of memory for a single acquisition trial was assessed after increasing retention intervals (see Fig. 2.4b, *below left*), namely, 15s, 1h, 3h and 6h, in Expt. 8; 15s, 15min, 30min and 1h, in Expt. 9; and 6h and 24h in Expt. 10. Retention intervals of 15s, 1h and 6h were tested repeatedly across experiments to compare data and detect possible variations in performance with repeated training of delayed

intervals (Strijkstra and Bolhuis, 1987). Rats were first given a block of 8 pretraining days. In the first 4 days the animals were trained with a fixed retention interval of 15s between trials 1 and 2. From days 5-8 the interval varied so that animals could get familiarized with the retention intervals tested in Expt. 8 (i.e. 15s, 1, 3 and 6h); a Latin square-type design was used to distribute the different retention intervals between groups of animals and pretraining days.

After pretraining, three different series of probe days (Expt. 8-10) assessed the effect of increasing retention intervals on one-trial memory strength. Each probe day was preceded by a pretraining day (inter-probe day). During inter-probe days the interval between trials 1 and 2 was either matched with the retention interval tested in subsequent probe days by each group of animals (Expt. 8-9) or fixed at 15s (Expt.10). The reason for using the same intervals in experiments 8-9 was to habituate the animals to each retention interval before testing, this was not possible in experiment 10 because giving intervals of 6h and 24h between trials 1 and 2 in inter-probe days would imply that only half of the animals would be trained the day before each probe day, compromising counterbalancing. In Expt. 10 each probe comprised two days; as it was not possible to retain animals in the watermaze room overnight they were moved back to the animal room after encoding trials (in both 6h and 24h conditions). In experiments 8-9 rats were kept in the watermaze room during retention intervals.

Experiment 11: Dependence of long-term place memory on number and temporal distribution of encoding trials (1)

Eighteen rats were used to investigate whether increasing the number and/or temporal distribution of encoding trials would strengthen long-term (6h) place memory in the watermaze. Memory strength was compared between encoding events comprising one trial, three massed (15s ITI) or spaced (10min) trials, and six massed or spaced trials. Memory for a single acquisition trial was further investigated when rats were allowed either 6s or 30s on the platform

at the end of the trial (see Fig. 3.9a). Rats were first pretrained for 10 days. The initial block of 4 pretraining days comprised an interval of 15s between trials 1 and 2. This interval was increased to 6h in the remaining pretraining days to familiarize the animals with the retention interval assessed in probe days. Days 7 and 10 of pretraining included probe trials to confirm that 6h memory for one encoding trial (30s on platform) had reached asymptotic levels previous to testing.

The experimental conditions were investigated in a series of 6 probe days. Each probe day was preceded by two pretraining days comprising an inter-trial interval of 6h between trials 1 and 2. Besides confirming the stability of performance over probe days, these pretraining days were particularly important to prevent carry over effects of memory between probe days in which different groups of animals were given different numbers of trials to the same platform location. Since 6h memory for a single encoding trial did not differ between experiment 8, in which rats were kept in the watermaze during the retention interval, and experiment 10, in which rats were moved back to the animal room (*see Results*), animals were kept in the watermaze room during the retention intervals. In subsequent experiments rats were only returned to the animal room when one or more experimental conditions included retention intervals of 24h or longer.

Experiments 12 and 13: Dependence of long-term place memory on number and temporal distribution of encoding trials

A new batch of 16 animals was used to re-examine the strength of memory for one and three spaced swim trials (10min ITI) after retention intervals of 6h (Expt. 12) and 24h (Expt. 13). After 4 pretraining days (4 trials/day; 15s ITI) rats were given 2 series of 2 probe days testing performance levels after a 6h retention interval (Expt. 12). This was followed by a third series of 2 probe days testing performance levels after a 24h retention interval. Probe days were

preceded by 2 inter-probe days with either 6h (Expt. 12) or 15s (Expt. 13) intervals between trials 1-2; an interval of 24h between trials 1-2 of inter-probe days would make it difficult to counterbalance the experimental design in Expt. 13, as in Expt. 10.

Experiment 14: Characterizing "weak" and "strong" encoding events

The aim of this experiment was to characterize "strong" and "weak" encoding events leading to the formation of either strong, i.e. long-lasting, memory, or weak, i.e. short-lasting, memory, in the DMP task in the watermaze. Memory for 3 spaced "swim" trials (10min ITI), in which rats were allowed 2min to swim to the platform and 30s on the platform, or 3 spaced "placement" trials (10min ITI), in which rats were solely placed on the platform for 30s, was assessed 30min and 24h after encoding, using the same batch of animals tested in experiments 12 and 13 (see Fig. 3.10a). Since animals had not been trained for a few weeks they were first given 4 pretraining days (4 trials/day; 15s ITI) to assess and re-establish levels of performance prior to testing (*data not shown*). After pretraining, performance for the above described experimental conditions was assessed in a series of 4 probes. Each probe comprised two days and was preceded by 2 pretraining days (4 trials/day; 15s ITI).

Experiments 15-17: Investigating behavioural analogues of "strong-before-weak" and "weak-before-strong" paradigms

Twenty animals were used to investigate if a "weak" encoding event, i.e. 3 spaced placement trials (10min ITI), would lead to the formation of memory detectable after 24h when preceded, or followed, within a time window, by a "strong" encoding event, i.e. 3 spaced swim trials (10min ITI). Weak and strong encoding events occurred in two different environments, i.e. two different watermazes located in different rooms ("upstairs" or "downstairs"; see *Apparatus* above), to reduce interference between generated memories. Rats were first given 12 pretraining

days (4 trials/day; 15s ITI), alternating between watermazes every day. A single set of 8 symmetrically distributed platform positions (e.g. A-H) was used for pretraining in both watermazes. The sequences of platform positions were designed so that it would be difficult to establish a learning rule based on the inner (I) or outer (O) location of the platforms not only within, but also between, watermazes [e.g. *G(I)*, B(O), *D(O)*, G(I), *A(I)*, D(O), *F(O)*, A(I), *C(I)*, F(O), *H(O)*, E(I); downstairs watermaze in italics]. Four platform locations were repeated between watermazes but no platform position was repeated in the design for a single watermaze. Start positions never matched for platform positions tested twice. These counterbalancing procedures were followed in all experiments using two watermazes simultaneously; platform positions were never repeated in experiments comprising 8 pretraining days.

Experiment 15 investigated if a weak encoding event (*target* event; upstairs watermaze) would produce memory detectable 24h later when preceded ("strong-before-weak" paradigm), or followed ("weak-before-strong" paradigm), 50min apart, by a strong encoding event (*modulatory* event; downstairs watermaze). Control conditions were included in the study, replacing strong "modulatory" events by weak encoding events (*control* events; downstairs watermaze) in both paradigms, to establish that it would be the "strong" nature of the "modulatory" event determining the emergence of potential synergistic interactions. Memory for strong "modulatory" events and weak "control" events was assessed 26h later (see Fig. 3.12a). These experimental conditions were investigated in a series of 4 probes. Each probe comprised 2 days and was preceded by 2 inter-probe days (4 trials/day; 15s ITI).

In experiments using two watermazes rats were always trained in the upstairs watermaze during inter-probe days and counterbalancing procedures for probe days were as follows. Probe days were conducted using two different sets of platform positions rotated 90 degrees between mazes. Platform positions were counterbalanced between animal groups and conditions within and between watermazes; throughout probe days, whenever platform position *X* was used in the upstairs watermaze to test a condition, platform position *Y* would always be used in the

downstairs watermaze for that same condition. *X* and *Y* configurations varied so that each animal equally experienced all possible transitions between inner and outer position rings. The time rats spent in each watermaze room before, between, and after, encoding trials was matched between watermazes; for example, in Expt. 15, the encoding events were separated by 50min, thus, rats were moved to the first watermaze room ~25min before the first encoding trial and remained in the same room for ~25 more minutes after the last encoding trial. After this time period rats were moved to the second watermaze room and the same procedure was repeated before they were taken back to the animal room for the remaining duration of the retention intervals. Rats were always moved back to the animal room between retention trials.

Experiment 16 tested short-term (30min) memory strength for weak "target" events when preceded, 50min apart, either by strong "modulatory" events or weak "control" events, in the "strong-before-weak" paradigm; memory for "target" events was also assessed after 24h, as in Expt. 15, in order to replicate previous findings and establish the consistency of performance between experiments (see Fig. 3.13a). Memory for "modulatory" and "control" events was assessed after 26h. Experimental conditions were tested in two different series of 4 probes; platform and start positions were changed between series. Probes comprised 2 days and were always preceded by 2 inter-probe days (4 trials/day; 15s ITI).

Experiment 17 investigated if reducing the time interval between encoding events in the "strong-before-weak" paradigm would facilitate the formation of long-term memory for target encoding events. "Modulatory" and "control" encoding were separated from target encoding events by 5min (see Fig. 3.14a). As in Expt. 15, memory strength for "modulatory" and "control" events was assessed 26h after encoding and memory for "target" events assessed 24h after encoding. The experiment comprised two probes (2 days *per* probe), each preceded by 2 inter-probe days (4 trials/day; 15s ITI).

Experiment 18: Dependence of long-term place memory for "strong" encoding events on protein synthesis in the hippocampus

Twelve rats were used to investigate the effect of bilateral intra-hippocampal infusions of the protein synthesis inhibitor anisomycin (125µg/µl; 1µl *per* side; 0.25µl/min) on the formation of long-term (6h) memory for strong encoding events in the watermaze (Fig. 3.15a). Rats were given infusions of anisomycin or vehicle (aCSF) 30min previous to encoding. Encoding comprised 3 standard swim trials separated by 5min. The reason for using an inter-trial interval of 5min in this experiment (as opposed to 10min used in previous experiments) was a technical constraint imposed by subsequent experiments designed to determine neuronal populations activated by two sequential events used in a "strong-before-strong" paradigm (see Expt. 20), using the catFISH technique (see Expt. 21). Pilot experiments showed that varying the inter-trial intervals from 5 to 10min did not alter the strength of long-term memory for 3 spaced swim trials (*data not shown*). Rats were first given 8 days of pretraining (4 trials/day; 15s ITI) and two series of 2 probe days to establish the absence of non-specific effects of infusion procedures on performance prior to testing (see *Supplement 8a-b*). A final series of 2 probe days tested the effects of anisomycin or vehicle infusions on performance. Each probe day was preceded by a pretraining day (4 trials/day; 15s ITI) and two additional training days followed the last probe day in order to assess if infusions of anisomycin produced permanent non-specific effects which would impact on performance.

Experiment 20: A behavioural analogue of the "strong-before-strong" paradigm

This experiment investigated if a "strong" encoding event occurring during protein synthesis inhibition in the hippocampus would lead to the formation of long-term memory if preceded, 60min apart, by a different "strong" encoding event occurring in a different environment. Rats were first given 8 pretraining days (4 trials/day; 15s ITI) alternating

watermazes every day. This was followed by a series of 2 probe days to establish the absence of mock infusion effects on performance (see *Supplement 10a-b*). Rats were then tested for four experimental conditions (see Fig. 3.17a). Conditions 1 and 2 comprised a replication of Expt. 18: aCSF or anisomycin (125µg/µl; 1µl *per* side; 0.25µl/min), were infused into the dorsal hippocampi 30min previous to a strong encoding event (target encoding event) and memory for this event was assessed after a 6h retention interval. The two remaining conditions introduced a second strong encoding event (the modulatory encoding event) occurring 30min previous to aCSF (*Cond. 3*) or anisomycin (*Cond. 4*) infusions; memory for this second event was assessed 8h after encoding. Each probe day was preceded by a pretraining day (4 trials/day; 15s ITI).

2.4.3. Electrophysiology

Experiment 7a compared the effects of hippocampal infusion of CNQX (*n*=7), AP-5 (*n*=7), and ACSF (*n*=6) on fast excitatory transmission evoked by low-frequency stimulation. A stable pre-infusion baseline was recorded for 20min before the start of the infusion. Recording continued for 2.5h after the start of the infusion. Experiment 7b compared the effects of hippocampal AP-5 and ACSF infusion on LTP. Stable pre-infusion baseline EPSPs evoked by low-frequency stimulation were recorded for 20min until infusion of AP-5 (*n*=6) or ACSF (*n*=6) started. Fifteen minutes after the start of the infusion, the perforant path was tetanized, low frequency test stimulation continued, and EPSPs were recorded for an additional 2.5h. To verify that AP-5 effects on LTP reflected interference with the induction but not the maintenance or expression of LTP, an additional group (post-AP-5, *n*=7) received hippocampal AP-5 infusion, not before tetanization but 5min after the end of the tetanus. The timing of the infusions in relation to the tetanus (i.e., 15min before the tetanus or 5 min after the tetanus) corresponded to the timing of the infusions in relation to the encoding phase in experiment 6.

2.4.4. *In situ* hybridization and immunohistochemistry

In collaboration with Dr. Almira Vazdarjanova's group at the Medical College of Georgia, cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH) and immunohistochemistry were used to: *i)* determine the extent of overlap obtained between populations of hippocampal CA1 neurons recruited by two "strong" encoding events occurring in different watermaze rooms, and *ii)* examine Arc protein expression in CA1 hippocampal neurons following strong encoding events in the watermaze.

Twenty animals were distributed in five different groups (4 per group) and tested in 5 different conditions (see Fig. 3.18a). Rats allocated to condition 1 were caged controls. These animals were sacrificed directly from their home cage and did not have any training in the watermaze. Rats allocated to conditions 3 and 5 were given two "strong" encoding events (3 swim trials; 5min ITIs), separated by 10min, and sacrificed 2-3 min after the last encoding trial. In condition 3 both encoding events occurred in the same watermaze room; in condition 5 each encoding event occurred in a different watermaze room. Conditions 2 and 4 were control conditions comprising animals that were moved to and between watermaze holding rooms exactly as rats in conditions 3 and 5 but that were not given any encoding trials. Each of these animals was paired and run with a "test" animal (Cond. 3 or 5) and sacrificed 1-2min after that animal. Animals allocated to conditions 2-5 were first trained in the DMP task. Training consisted of 8 days of pretraining (4 trials/day; 15s ITI), alternating between watermazes, and two probe days, each testing long-term memory for two strong encoding events occurring in two different watermazes 60min apart. The purpose of this design was to match the extent of training given to rats used in Expt. 20 previous to testing, compare levels of performance between experiments, and compare levels of performance obtained in the upstairs and downstairs watermazes (see *Behavioural data* in *Supplement 11*). Distribution of previously trained rats in conditions 2 to 5 matched the levels of performance obtained in probe days between animals allocated to each condition. All rats were sacrificed on day 11.

2.5. Intra-hippocampal infusions

2.5.1. Implantation of infusion canullae

One day before until 3 days after surgery, animals received an analgesic in their drinking water (Rimadyl Large Animal Solution; 2ml/L). Anesthesia was induced with 5% halothane and maintained with 1–3% halothane, delivered in oxygen. The rats were placed in a stereotaxic frame, the scalp was incised to expose the skull, and bregma and lambda were aligned in the same horizontal plane. Infusion guide canullae (26 gauge) with stylets (33 gauge; C315; Plastics One, Bilaney, UK), which should prevent occlusion of the guides and which were secured to them by plastic caps, were implanted through small holes drilled into the skull. The stylet tips projected 0.5mm from the end of the guide and were aimed at the following coordinates above the posterior dorsal hippocampus: 4.5mm posterior and 3.0mm lateral from bregma and 3.0mm ventral from the dura. These coordinates were used in previous studies examining the effects of hippocampal micro-infusions in different memory tasks in our laboratory (Riedel et al., 1999; Steele and Morris, 1999; Day et al., 2003). Guide cannulas were fixed to the skull with dental cement and stainless steel screws. After surgery, rats had a recovery period of 7–10 days before the start of any other procedure. During this period, the rats were handled and habituated to the restraint necessary for hippocampal infusions. Rats used in experiment 7 were only implanted with one infusion guide cannula into the left hemisphere. The plastic pedestal of the guide cannulas was partly cut away before implantation, and the locations for insertion of the stimulating and recording electrodes (see below) were marked on the skull and kept free of dental cement.

2.5.2. Infusion procedures

For behavioural experiments, rats were restrained manually and given simultaneous bilateral intra-hippocampal infusions. The stylets in the guide cannulas were replaced by infusion cannulas (33 gauge; C315; Plastics One) connected to micro-syringes in a micro-infusion pump via flexible polyvinyl chloride tubing. The tips of the infusion cannulas projected 0.5mm beyond the end of the guides. A volume of 1µl per cannula was infused at a rate of 0.4µl/min in the experiments in the "event arena" task and 0.25µl/min in the experiments in the watermaze. To allow for absorption of the infusion bolus by the brain tissue, the infusion cannulas were left in place for 1min before being replaced by the stylets. Mock infusions were conducted with empty syringes and tubing, other than this, infusion procedures were as described above. For the electrophysiological study (Expt. 7), rats were first anaesthetized and the Plastics One stylets were replaced by stylets without a plastic cap to make space for the electrodes and to prevent occlusion of the guide cannulas until infusion. Otherwise, infusion procedures were identical to those of the behavioural experiments in the "event arena". In the autoradiography study (Expt. 21), the infusion procedures followed the same protocol as the behavioural experiments in the watermaze.

2.6. Electrophysiology

Electrophysiological procedures were similar to previous studies (Martin, 1998; Riedel et al., 1999). The rats were anesthetized with urethane (1.5g/kg, i.p.), and their rectal temperature was maintained at 36.2±0.2°C. Ipsilateral to the infusion guide, they were stereotaxically implanted with a twisted bipolar stimulating electrode (distance between electrode tips, 0.5–1mm) into the angular bundle of the perforant path and a monopolar recording electrode into the hilus of the dentate gyrus. Electrodes (Teflon-coated platinum90-iridium10 wire; outer

diameter, 112μm) were aimed at the following coordinates (in mm): stimulating electrode: 7.5 posterior and 4.0 lateral from bregma and 2.5 ventral from the dura; recording electrode: 3.5 posterior and 2.0 lateral from bregma and 3.0 ventral from the dura. Recordings were made against a reference electrode placed on the cortex anterior to bregma. Stimulation was applied via a Neurolog stimulus isolator (NL800; AutoMate Scientific, San Francisco, CA). Field EPSPs were amplified and filtered (1 Hz low-frequency cutoff, 5 kHz high-frequency cutoff) by a differential AC amplifier (model 1700; AM Systems, Everett, WA). A personal computer running dedicated Labview software was used to control the stimulation and to record (20,000 Hz sampling frequency) and analyze EPSPs. The main measure of the evoked response was the slope of the initial rising part (2.0 –2.6 or 2.2–2.8ms after stimulation) of the EPSP. Electrodes were initially positioned 1mm dorsal to the target coordinates. Biphasic 0.2ms, 0.5 mA stimulation was delivered at 0.1 Hz, and the final coordinates of the electrodes were adjusted to record a positive EPSP and to maximize its slope. The slope was aimed to be at least 2 mV/ms under these conditions, and occasionally the stimulus intensity was increased to maximally 1 mA to reach this value. After positioning the electrodes, low-frequency test stimulation continued with biphasic 0.1ms pulses of the same intensity, delivered at 0.05 Hz for the rest of the experiment. Tetanus to induce LTP consisted of three trains of 50 biphasic 0.2ms pulses at 250 Hz with 60s between trains (overall 2min). At the end of the experiments, the locations of the electrode tips were marked by a 10mA, 2s biphasic pulse to the electrodes and, as described for experiment 3, rats were perfused and their brains were further processed to verify cannula and electrode placements.

2.7. [^{14}C] L-leucine autoradiography

2.7.1. Protocol

In collaboration with Dr. Paul Kelly and Dr. Harry Olvermann at the Centre for Cognitive and Neural Systems, autoradiographic imaging and quantitative densitometric analysis of [^{14}C] L-leucine uptake into the brain (Smith, 1991) were used to assess the magnitude, diffusion and temporal decay of protein synthesis inhibition following intra-hippocampal infusions of anisomycin. Anisomycin and aCSF were infused simultaneously in opposed hippocampi so that paired measures could be obtained from each individual animal. Other than this, the infusion protocol was identical to that used in experiment 18 (see Fig. 3.16). A bolus of [^{14}C] L-leucine (Amersham Biotech; specific activity 59 mCi.mmol^{-1}) was injected into the tail vein (7.5 μCi.100g^{-1}) 30min (n=6), 3h45min (n=4), 6h45min (n=4) or 24h:45 (n=4) after the start of infusions. One hour after the injection of leucine, rats were decapitated and trunk blood collected into heparinized centrifuge tubes. Brains were frozen in 2-methylbutane at -45ºC, mounted onto specimen holders with embedding medium (Lipshaw), and stored overnight at -80ºC. Whole-blood samples were centrifuged (13000xg for 60s) and 20μl aliquots of plasma were taken for liquid scintillation analysis to determine blood concentrations of tracer at the end of the experiments. The brains were sectioned (20μm) in the coronal plane using a cryostat maintained at -22ºC. Three consecutive sections from every 100μm cut throughout the rostro-caudal axis of the hippocampus were thaw mounted onto glass coverslips and rapidly dried on a hot plate (75ºC). In areas of the brain more anterior and posterior to hippocampus, three sections were collected from every 400μm of tissue sectioned.

2.7.2. Image acquisition and analysis

Autoradiograms were prepared by applying the sections, together with a series of eight pre-calibrated [^{14}C]-standards (40–1069 nCi/g tissue equivalents: Amersham Biotech, UK), to X-ray film (Kodak, SB-5) in light-tight cassettes, for 7 days. Films were processed after this exposure period in accordance with manufacturers instructions. Sections adjacent to those used for autoradiography were stained with cresyl-violet. Autoradiograms were analysed using a computer based image analysis system (MCID/M5+). The background density of the films was measured, and local tissue isotope concentrations were derived from the optical density of autoradiographic brain images relative to the [^{14}C]-standards, following background subtraction. Measurements of tracer levels in hippocampal subfields were taken from three sets of consecutive sections at the level of the habenula (bregma -3.30mm approx.) and the medial geniculate (bregma -5.80mm approx.). Thus, for each subfield at each level, tracer levels were derived from the mean of nine measurements for each side of the brain separately. To determine the concentrations of tracer found in the hippocampus as a whole, the outline of the structure was delineated using cresyl violet sections, the area stored on the computer, and then superimposed upon the adjacent autoradiographic images.

2.8. *In situ* hybridization and immunohistochemistry

2.8.1. Brain dissection and sectioning

After decapitation, the brains were rapidly removed (≤ 2min), flash frozen in isopentane (at approximately -50°C), and stored at -80°C. A tissue section comprising the most caudal 8mm of the diencephalon was cut from each of the frozen brains of each animal for cryosectioning. Each of these sections was mounted in individual blocks using Neg 50 frozen section medium

(Richard-Allan Scientific, Kalamazoo, MI), such that one brain from each condition was present in each block. The blocks were cryosectioned into 20µm-thick coronal sections at -18°C, captured on Superfrost/Plus slides, and stored at -20°C.

2.8.2. Fluorescence *in situ* hybridization

Slides containing areas of the dorsal hippocampus (~3.1-3.6 mm posterior to Bregma) were selected from each block and stained for *Arc* and *Homer 1a* mRNA according to the catFISH method described in detail elsewhere (Guzowski and Worley, 2001). Briefly, the tissue was fixed in 4% paraformaldehyde, washed in 2X sodium chloride/sodium citrate buffer (SSC), and placed in a 0.5% acetic-anhydride solution, followed by 50:50% acetone:methanol. After a pre-hybridization step, the tissue was hybridized with an antisense *Arc* and antisense *H1a* mRNA probe (100 ng/slide) tagged with digoxigenin or DNP diluted in hybridization buffer (Sigma, St. Louis, MO) overnight at 56°C. After a series of washes, including treatment with RNase A, endogenous peroxidase activity was blocked with 2% H_2O_2. The slides were incubated for 2h with anti-digoxigenin peroxidase-conjugated antibody (Roche Products, Hertfordshire, UK, 1:500) and the stain was visualized using the CY3 TSA fluorescence system. Following extensive quenching of peroxidase activity with 2% H_2O_2 and a series of washes, an anti-DNP peroxidase-conjugated antibody was applied (Zymed, Carlsbad, CA) at 1:200 and incubated at room temperature for 2h. The slides were then washed and the labeling was revealed with the FITC TSA fluorescence system. The nuclei were counterstained with DAPI (Invitrogen) nucleic acid stain and coverslipped with Vectashield to protect the fluorescence.

2.8.3. Immunohistochemistry

Slides from tissue adjacent to the ones chosen for *in situ* hybridization were stained for Arc protein. The tissue was fixed in 2% paraformaldehyde, pH 7.3, for 10min and then washed twice in 2X SSC, pH 6.8, for 10min each followed by 50:50% acetone:methanol at 4°C for 7min. The tissue was then washed in 2X SSC and 0.05% Tween 20 and quenched in 1%H_2O_2 in 2X SSC for 20min. Endogenous biotin was blocked using the Zymed Avidin/Biotin Blocking Kit (Invitrogen, Carlsbad, CA) followed by blocking with tyramide signal amplification kit (TSA) blocking buffer (PerkinElmer Life Sciences, Waltham, MA) with 1.5% normal goat serum. Slides were incubated in polyclonal rabbit anti-Arc antibody (1:2000) supplied by Dr. Paul Worley's laboratory for 72h at 4°C. Incubation with the anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30min at room temperature was followed by amplification with the ABC avidin-biotin system (Vector Laboratories) for 1h. The staining was visualized using the cyanine 3 (CY3) TSA fluorescence system (PerkinElmer Life Sciences), and the nuclei were counterstained with SYTOX Green (Invitrogen) or DAPI (Invitrogen) nucleic acid stains.

2.8.4. Image acquisition and analysis

Series of image stacks (z-stacks) from CA1 were collected with a 25x objective on a Zeiss AxioImager/ Apotome system. During image collection, excitation source intensity and exposure settings were optimized and kept constant for all brains. Unbiased stereological cell counting and classification were applied as follows: From within the channel with nuclear staining neurons were segmented. Putative glial cells, those with small, intensely, and uniformly stained nuclei, were excluded from the analysis. With all nuclear and IEG staining channels turned on, segmented neurons were classified with Axiovision imaging software (Zeiss) using an optical dissector method, which minimizes sampling errors attributable to partial cells and

stereological concerns, because variations in cell volumes do not influence sampling frequencies (West, 1999). Pairs of adjacent "lookup" and "sample" sections were stacked so that the first optical section in a stack was the first "lookup section," and the second optical section was the first "sample section" as well as the "lookup section" for the next "sample section." The dissector consisted of all "sample sections" in the top 60% of a given stack. The dissector counting rule instructed that all neuron-like cells with leading edges present in the dissector should be selected. This rule ensured that all neurons had equal probabilities of being included in the samples, regardless of their size, as each of them was defined by a point (their top) rather than volume. This rule also minimized type I classification errors. Cells were classified as Arc^+ or $H1a^+$ when *Arc* or *H1a* mRNA at the foci of transcription was present on at least three planes. Cells with both were classified as $Arc^+/H1a^+$. Cells that did not meet these criteria were classified as 'negative'. The percent of cells with IEG expression initiated by the first encoding event was calculated as follows: ($H1a^+$ + $Arc^+/H1a^+$)/total number of cells. The percent of cells with IEG expression initiated by the second encoding event was calculated as follows: (Arc^+ + $Arc^+/H1a^+$)/total number of cells. Cells were classified as Arc^+ when cytoplasmic Arc protein was visible around two-thirds of the nucleus on at least three optical planes. All analyses were done by an experimenter blinded to the group designations of the brains from which the image stacks were collected.

2.9. Drugs

Phosphate-buffered artificial cerebrospinal fluid (aCSF) (in mM: 150 Na^+, 3 K^+, 1.4 Ca^{2+}, 0.8 Mg^{2+}, 155 Cl^-, 0.2 H2PO4$^-$, 0.8 HPO4^{2-}, pH 7.2) was made using pyrogen-free (injectable) water and used as infusion vehicle or for control infusions. Drug concentrations for infusions were 0.89µg/µl of the competitive AMPA/kainate receptor antagonist CNQX (disodium salt; $C_9H_2N_4O_4Na_2x1H_2O$; Tocris, Bristol, UK), 5.9µg/µl of the competitive NMDA receptor

antagonist D-AP5 ($C_5H_{12}NO_5P$; Tocris), and 125µg/µl of the protein synthesis inhibitor Anisomycin ($C_{14}H_{19}NO_4$; Sigma). The solution of CNQX was facilitated by slight sonification. Anisomycin was dissolved in HCl and diluted with aCSF. The pH of the drug solutions was adjusted to 7.2 by addition of concentrated phosphoric acid (for CNQX) or 1M NaOH (for D-AP5 and Anisomycin). Drug solutions were prepared in large quantities and divided into aliquots, kept frozen at -20°C until use. The CNQX solution was slightly sonicated after thawing.

2.10. Histology

At the completion of the experiments, rats were anesthetized with an overdose of Euthatal (Harlow, Essex, UK) and perfused transcardially with saline, followed by 4% formaldehyde solution to fix the brain tissue. Brains were extracted from the skull, post-fixed in 4% formaldehyde solution, and cut into 30 µm coronal sections on a freezing microtome. Every third section through the area of interest was mounted on slides and stained with cresyl violet. The sections were examined with a light microscope under 20-fold magnification to verify cannula placements and draw them onto plates from a rat brain atlas (Paxinos and Watson, 1998).

2.11. Statistical analysis

Behavioural analysis - Paired Student's *t* tests or repeated-measures ANOVA were used to analyze the influence of within-subjects variables on behavioural measures, unless stated otherwise. Fisher's protected least significant difference (PLSD) post hoc tests were used to further examine main effects of the ANOVA. Paired Student's *t* tests were used to compare

performance measures to expected values based on chance. Pearson product-moment correlation coefficients were used to compare performance values obtained by the same animals in the upstairs and downstairs watermazes in conditions involving two different encoding events. The relative standard error of the mean of the different measures of performance was calculated as the standard error of the mean divided by the absolute value of the mean and multiplied by 100. Normalization of the watermaze data to chance was calculated as the percentage of time swimming in the correct zone divided by chance level (12.5%). Normalization of the event arena data to chance was calculated as the percentage of time digging in the correct sandwell divided by chance level (20%). Planned unpaired Student's *t* tests were used to compare performances in the event arena and the watermaze for the same retention intervals.

Electrophysiology (Expt. 7) - EPSP slopes were averaged in 5min blocks and expressed as a percentage of the mean slope during the 20min baseline recording (percentage of baseline EPSP slope). Student's *t* tests or ANOVA were used to analyze the influence of within- and between-subject variables.

Autoradiography (Expt. 19), in situ hybridization and immunohistochemistry (Expt. 21) - Expt. 19: Paired Student's *t* tests were used to compare tracer concentration values between hemispheres. Expt. 21: Differences between groups were assessed by means of ANOVA. Fisher's protected least significant difference (PLSD) *post hoc* tests were used to further examine main effects of the ANOVA. The level of significance was set at $p \leq 0.05$. Data are presented as Mean ±1 SEM.

Chapter 3: Results

3.1. Studies of one-trial place memory in a new event arena task

3.1.1. Task acquisition (Expt. 1-2)

Experiment 1

The rats that had previous training in the flavour–place memory task rapidly learned the win–stay rule of the place memory task. During the encoding phase, they searched the arena for the single open sandwell, with their heads near the arena floor, whereas in the retrieval phase, they moved relatively quickly to the correct place. This different pattern of movement is suggestive that retrieval was guided by spatial memory. After a few training trials, first choices were significantly above and errors below chance during the retrieval phase (Fig. 3.1, left and centre, gray symbols). The percentage of rats making correct first choices increased over the 18 trials (linear regression, percentage first choices vs. day; slope, 1.74; r=0.64; p<0.004) (Fig. 3.1, left, gray symbols). When collapsed into 3-trial blocks the percentage of correct first choices for

each rat was significantly higher than chance level (20%) for the last three blocks [trial 10-12: 48.9±8.5%, trial 13-15: 37.8±7.9%, trial 16-18: 53.3±10.2%; $t_{(14)}>2.2$; $p<0.05$], but not for the first three blocks [trial 1-3: 24.4±7.6%, trial 4-6: 28.9±7.2%, trial 7-9: 31.1±6.1%; $t_{(14)}<1.9$; $p>0.08$]. The mean number of errors decreased throughout the 18 trials ($F_{(17,238)}=2.02$; $p<0.02$) and was significantly lower than chance for trials 4, 5, and 7–18 ($t_{(14)}>2.5$; $p<0.02$) but not trials 1–3 and 6 ($t_{(14)}<1.5$; $p>0.16$) (Fig. 3.1, centre, gray symbols). During the retrieval phase of the probe trial (trial 17), the percentage of dig time at the sandwell in the correct location was nearly four times as high as the average at the sandwells in the four novel locations ($t_{(14)}=4.9$; $p<0.001$) (Fig. 3.1, right, gray symbols). Dig time in the correct sandwell was higher and the average dig time at the novel sandwells was lower than chance ($t_{(14)}>4.9$; $p<0.001$).

Experiment 2

This experiment used a new batch of animals to see if the levels of performance obtained in Expt. 1 could be replicated during the acquisition of the task (Fig. 3.1, compare black and gray symbols). After shaping and habituation, the otherwise naive rats learned the task within a few trials. The percentage of rats making correct first choices increased over the 17 trials [linear regression, percentage first choices vs. day: slope 1.16, R=0.45, p=0.07] (Fig. 3.1, left, black symbols). If the percentage of correct first choices was calculated in five 3-trial blocks (trial 1-15) and one last 2-trial block (trial 16-17) for each rat, the mean percentage of correct first choices did not differ from chance level (20%) for the first 3-trial block [trial 1-3: 24.4±7.6%; $t_{(15)}<1$], but was significantly higher than chance for all subsequent trial blocks [trial 4-6:37.5±7.4%, trial 7-9: 47.9±8.6%, trial 10-12: 41.7±5.7%, trial 16-17: 46.9±10.7%; $t_{(15)}>2.3$; $p<0.05$], except for the penultimate one [trial 13-15: 37.5±9.6%; $t_{(15)}=1.83$; p=0.09]. The mean number of errors decreased throughout the 17 trials, even though this effect failed to reach statistical significance [$F_{(16,240)}=1.4$, p=0.14]. The mean error was significantly lower than

chance level for trials 5 and 9-17 (including the probe trial, trial 17) [$t_{(15)}$>2.4, p<0.05], but not for trials 1-4 and 6-8 [$t_{(15)}$<2.0, p>0.05] (Fig. 3.1, centre, black symbols). In the probe trial, the percentage of dig time at the sandwell in the correct location was more than four times as high as the average at the sandwells in the four novel locations [$t_{(15)}$=5.7, p<0.0001] (Fig. 3.1, right, black symbols). Dig time at the correct sandwell was higher, and average dig time at the novel sandwells lower, than chance [$t_{(15)}$>5.6, p<0.0001]. The comparison of the two data sets with an overall ANOVA, with experiment as a between-subjects factor, did not reveal any significant main effect of experiment nor an interaction of experiment with any of the within-subjects variables (F<1).

3.1.2. Dependence of one-trial memory on visuo-spatial information (Expt. 3-4)

Arena rotation between encoding and retrieval phases (Expt. 3)

Performance was normal when the arena was rotated between encoding and retrieval phases, showing that rats did not rely on odour traces on the arena surface. At both retention intervals tested, 20 and 360min, dig time significantly differed between correct and novel sandwells and was higher than chance at the correct sandwell and lower than chance at the novel wells ($t_{(15)}$>2.5; p<0.05). The dig-time measure also revealed forgetting over time (Fig. 3.2a). ANOVA of the percentage of dig time (average of two probe trials at both retention intervals) at the different sandwells revealed a significant interaction of retention interval by sandwell type (correct or novel) ($F_{(1,15)}$=15.1; p<0.005): dig time at the correct well was lower and dig time at the novel wells was higher at 360min than at 20min ($t_{(15)}$>3.9; p<0.005). The dependence of performance on retention interval was further suggested by the first-choice and error measures; however, the statistical analysis of these measures was less conclusive (Supplement 1).

Figure 3.1: Acquisition of the one-trial place memory task in the “event arena” (Expt. 1-2)

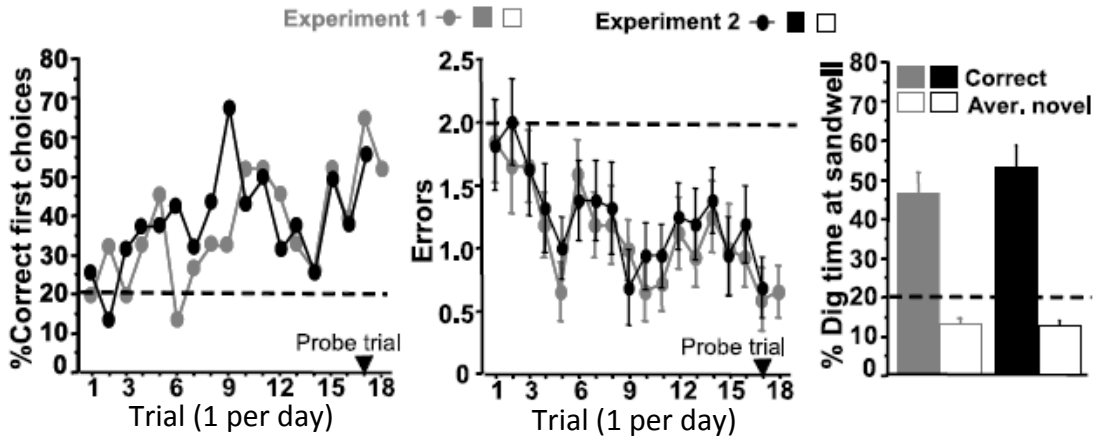


Figure 3.1. Acquisition of the one-trial place memory task in the “event arena” (Expt. 1-2). Stippled horizontal lines indicate chance values of performance measures. The rats in experiment 1 (gray symbols; n=15) were previously trained on a flavour-place memory task, whereas the rats in experiment 2 (black symbols; n=16) were only shaped to dig in sandwells and habituated to the arena before training on the place memory task. The percentage of correct first choices (percentage of rats digging first in the correct sandwell; *left*) and errors (number of novel wells in which rats dug before digging in the correct one; mean ±1 SEM; *centre*) for the initial training trials conducted with a 5min retention interval. Trial 17 was a probe trial, in which food was omitted during the retrieval phase and the rats’ dig time was measured for 60s to calculate the percentage of dig time at the correct sandwell and the average percentage of dig time at novel sandwells (mean ±1 SEM; *right*).

Darkness during retrieval (Expt. 4)

Darkness during the retrieval phase completely disrupted performance, demonstrating the requirement of visuo-spatial information. When it was dark during the retention interval but light during the retrieval phase, all rats showed good performance. However, when it was dark during the retrieval phase, 4 rats did not dig in any sandwell, and the other 12 rats dug without discrimination between correct and novel sandwells ($t_{(11)}<1.3$; $p>0.25$) (Fig. 3.2b). The former four rats performed well in the light (first choices, three of four rats; errors, 0.75±0.75; for dig time see Fig. 3.2b, inset); a formal statistical analysis of performance measures is not presented because of the small number of rats. For the 12 rats digging both in darkness and in light, ANOVA of the dig-time measure revealed a significant interaction between the lighting condition in the retrieval phase (dark or light) and the sandwell type (correct or novel) ($F_{(1,11)}=11.6$; $p<0.01$). Additional comparisons revealed a higher proportion of dig time at the correct well and a lower proportion at novel wells in light compared with darkness ($t_{(11)}>3.4$; $p<0.01$). Only in light, but not in darkness, did the percentage of dig time significantly differ between correct and novel sandwells and was higher than chance at the correct sandwell and lower than chance at the novel wells (light: $t_{(11)}>7.2$; $p<0.0001$; darkness: $t_{(11)}<1.3$; $p>0.24$). Error and first-choice measures also supported the view that rats are unable to discriminate between correct and novel sandwells in darkness, but strong statistical conclusions were hampered because there was only one trial for each condition and only 12 rats contributed data for both conditions (*data not shown*).

3.1.3. Persistence of one-trial place memory (Expt. 5)

One-trial place memory strength declined monotonically with increasing retention intervals. This was particularly evident with the dig-time measure obtained during the probe trials at each of the four retention intervals. Analysis of the percentage of dig time (average of

two probe trials per retention interval) at the different sandwells revealed a highly significant interaction of retention interval by sandwell type (correct or novel) ($F_{(3,45)}$=17.3; p<0.0005): while dig time at the correct well decreased with increasing retention intervals ($F_{(3,45)}$=17.3; p<0.0001), the average dig time at novel wells increased ($F_{(3,45)}$=10.2; p<0.0001), reflecting weaker memory for the correct location at longer retention intervals (Fig. 3.3a). Compared with the 5min retention interval, dig time decreased at the correct and increased at the novel sandwells at all other retention intervals tested (60, 180, and 360min; p<0.005). Dig times at both correct and novel sandwells also differed between 60 and 360min retention intervals (p<0.05). Nevertheless, at all retention intervals tested, including 360min, the percentage of dig time was significantly higher at correct than at novel sandwells, as well as higher than chance at the correct sandwell and lower than chance at the novel wells ($t_{(15)}$>2.4; p<0.05). Thus, forgetting takes place over time but memory can still be detected after 6h.

Analysis of the first-choice and error measures also indicated reduced performance with increasing retention intervals; however, statistical analysis did not reveal a significant influence of retention interval variation (Fig. 3.3b). The ANOVA of the percentage of first choices averaged over 6 trials (4 standard and 2 probe trials; Fig. 3.3b; white) at each of the four retention intervals did not show a significant effect of retention interval [$F_{(3,45)}$<1], even though the percentage of first choices decreased at 360 minutes when compared to the other retention intervals. ANOVA of the number of errors averaged over 6 trials (4 standard and 2 probe trials; Fig. 3.3b; black) at each of the four retention intervals only revealed a trend for an effect of retention interval [$F_{(3,45)}$=2.32, P=0.09]. The average first-choice and error measures significantly differed from chance at all intervals [$t_{(15)}$>3.5, P<0.001]. The absence of an interval effect in the analysis of first-choice and error measure was not due to the possible use of olfactory cues emanating from the food reward during standard trials: there was also no significant retention interval effect on first-choice and error measure if only the probe trials were analyzed [$F_{(3,45)}$<1]. Finally, it is worth noting that the performance measures obtained at a

retention interval of 360min were not different between this experiment and experiment 3 (p>0.8). Measures obtained at 20min in Expt. 3, in particular the dig-time measure, were also within the range observed between 5 and 60min retention intervals in this experiment.

Relative variability of performance measures

From experiment 1 to 5, the new protocol presented robust and statistically reliable above-chance measures of one-trial place memory for discrete trials. First-choice, error, and dig-time measures reflected statistically significant performance. Although both error and dig-time measures are continuous values for discrete trials, the dig-time measure was the most consistent measure of one-trial place memory. It showed relatively little inter-individual variance (relative SEM in a discrete trial, ~10%, compared with 30% for the error measure) and little variation between trials with different sets of correct and novel sandwells. Notably, the sensitivity of the dig-time measure as an indicator of memory strength was particularly evident with its gradual alteration with increasing retention intervals. Based on the results of experiments 1-5, analysis of experiment 6 will mainly focus on the dig time measure.

Figure 3.2: Dependence of one-trial place memory on visuo-spatial information (Expt. 3-4)

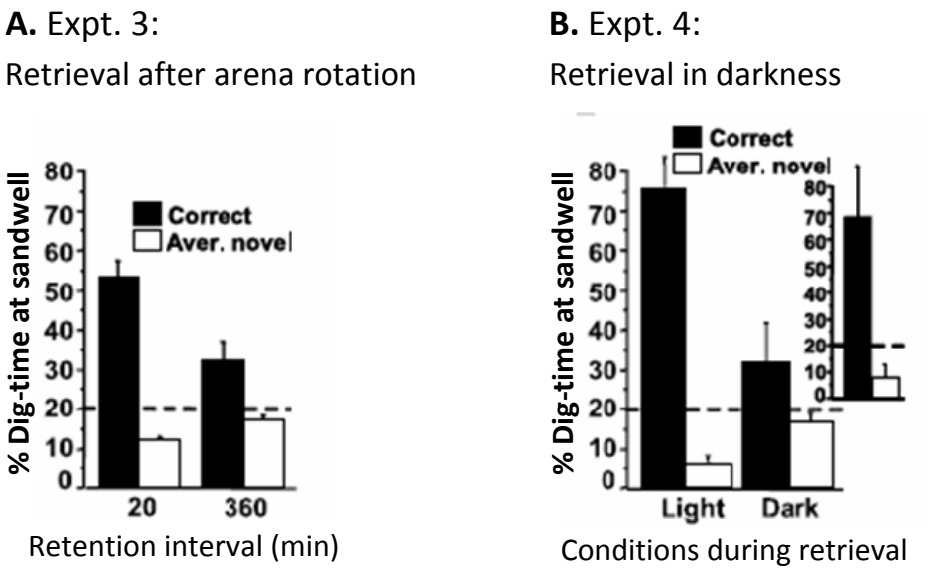


Figure 3.2. Dependence of one-trial memory on visuo-spatial information (Expt. 3-4). ***A)*** Arena rotation between encoding and retrieval phases (Expt. 3). To establish that performance did not rely on cryptic odor cues, rats (n=16) were tested after the arena was rotated between encoding and retrieval phases. Retention of memory was investigated 20 and 360min after encoding. The dig-time measure is shown. Above chance performance was observed for the correct sandwell but not for the average novel sandwells. Stippled horizontal lines indicate chance value of the performance measure. Mean±1SEM. ***B)*** Darkness during retrieval (Expt. 4). To establish the dependence of performance on visuo-spatial cues, retrieval in darkness was compared to retrieval in light after a retention interval of 20min. The dig-time measure is shown for both conditions. Only 12 rats dug under both conditions (main graph), whereas four rats did not dig in any sandwell during darkness but performed normally in light (inset). That the average percentage of dig time in the dark condition (right; 32.3±10.1%) is numerically higher than chance is essentially attributable to a single rat that dug briefly (1.0 s) in the correct well, without touching novel wells. Mean±1SEM.

Figure 3.3: Persistence of one-trial place memory in the "event arena" task (Expt. 5)

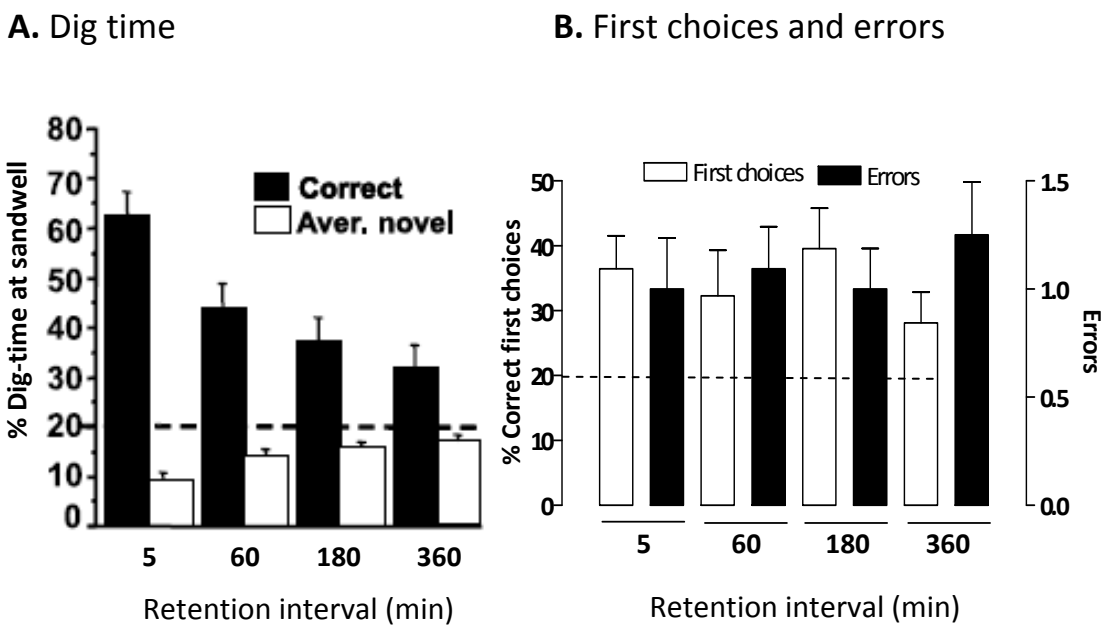


Figure 3.3. Persistence of one-trial place memory in the "event arena" task (Expt. 5). One-trial place memory was tested after retention intervals of 5, 60, 180, and 360min. ***A)*** Dig-time measures for the probe trials at the four retention intervals (average of two probe trials). ***B)*** The percentage of correct first choices (white) and the number of errors (black) are shown (six trials at each retention interval: four standard training trials and two probe trials). Stippled horizontal lines indicate chance values for dig-time (A) and first choice (B) measures of performance. Mean±1SEM (n=16).

3.1.4. Contribution of hippocampal glutamate receptor activation to encoding and retrieval of one-trial place memory (Expt. 6-7)

The relevance of hippocampal NMDA and AMPA receptors for encoding and retrieval of one-trial place memory was tested by specifically blocking these receptors during different phases of the one-trial place memory task. We first tested the effects of hippocampal NMDA receptor blockade by D-AP5 on both encoding and retrieval and then the effects of AMPA receptor blockade by CNQX on retrieval (Expt. 6). Since different effects on hippocampal synaptic transmission and on synaptic plasticity might underlie distinct effects of hippocampal D-AP5 and CNQX infusions on encoding and retrieval of one-trial place memory we further examined the effects of hippocampal D-AP5 and CNQX infusions, conducted exactly as in experiment 6, on synaptic transmission and long-term potentiation (LTP) at one intrinsic hippocampal connection, the perforant-path dentate gyrus synapses, in anesthetized rats (Expt. 7). In order to establish that the rats used in experiment 6 showed robust place memory at a 5min retention interval (as observed in Expt.1) and at longer retention intervals (required for infusion studies examining retrieval mechanisms) their performance was assessed after 5 and 45min retention intervals, previous to surgery. Above chance performance was observed with both retention intervals (see *Supplement 2a*). After surgery, a series of probe trials established the absence of mock infusion effects on performance assessed at a 20min retention interval (see *Supplement 2b*).

Encoding, but not retrieval, of one-trial place memory depends on activation of NMDA receptors in the hippocampus (Expt. 6)

Hippocampal infusion of the NMDA receptor antagonist D-AP5 impaired encoding but not retrieval of one-trial place memory (Fig. 3.4a). An overall ANOVA of the percentage of dig time at the different sandwells revealed a highly significant interaction between the infusion condition and the sandwell type (i.e., correct or novel; $F_{(3,39)}$=6.8; p<0.001). Subsequent separate ANOVAs on the percentage of dig time spent at the correct sandwell, as well as on the average percentage of dig time at the sandwells in novel locations, revealed significant main effects of the infusion condition. When rats received bilateral intra-hippocampal infusions of D-AP5 before encoding, the percentage of dig time spent at the correct sandwell was significantly lower, whereas the average percentage of dig time spent at the sandwells in novel locations was significantly higher (p<0.01), than in the other three conditions, which did not differ (p>0.16). Moreover, when rats received D-AP5 before encoding, the percentage of dig time in correct and novel locations did not differ from chance ($t_{(13)}$<1.2; p>0.25). In all other infusion conditions, the percentage of dig time at the correct sandwell was higher than the average percentage of dig time in the novel locations, with the former being significantly higher and the latter being significantly lower than the chance level ($t_{(13)}$>5.1; p>0.0005). Although the average number of errors was higher when D-AP5 was infused before encoding (1.36±0.31) compared to D-AP5 infusion before retrieval (0.71±0.24), and aCSF infusion before encoding (1.00±0.28) or retrieval (0.86±0.28), the ANOVA did not yield a main effect of infusion condition on the error measure ($F_{(3,39)}$= 1.0). However, the average number of errors did not differ significantly from chance when rats received D-AP5 before encoding ($t_{(13)}$=2.1; p>0.05), but it was lower than chance in all other conditions ($t_{(13)}$>3.6; p<0.005) (*data not shown*).

Retrieval of one-trial place memory depends on activation of AMPA receptors in the hippocampus (Expt. 6)

Hippocampal CNQX infusion impaired the retrieval of one-trial place memory (Fig. 3.4b). An overall ANOVA of the percentage of dig time spent at the different sandwells revealed a highly significant interaction between the infusion condition and the sandwell type ($F_{(1,13)}$=23.4; p<0.0005). Subsequent paired t tests revealed that, compared with the ACSF control condition, dig time at the correct sandwell was decreased and dig time at the sandwells in novel locations was increased ($t_{(13)}$>4.8; p<0.0005) when rats received hippocampal CNQX infusion before retrieval. Nevertheless, in both the aCSF and the CNQX infusion conditions, the percentage of dig time at the correct sandwell was higher than the average percentage of dig time in the novel locations ($t_{(13)}$>3.6; p<0.003), with the former being significantly higher and the latter being significantly lower than the chance level ($t_{(13)}$>3.6; p<0.003). The number of errors was significantly increased in the CNQX (1.36±0.25) compared with the aCSF (1.00±0.021; $t_{(13)}$=2.7; p<0.02) condition, although it was significantly lower than chance in both groups ($t_{(13)}$>2.5; p<0.02).

Non-mnemonic infusion effects

The effects of the hippocampal drug infusions on non-mnemonic behavioural processes possibly necessary for task performance were assessed by careful observation of the rats' behaviour, as well as by analysis of the absolute dig time during the retrieval phase. Hippocampal infusion of D-AP5 often resulted in slight ataxia (i.e., slight unsteadiness when moving), which lasted for ~15–20min. Inspection of one-trial place memory performance in individual rats after hippocampal D-AP5 infusion did not indicate a relationship between the occurrence or absence of ataxia and performance. CNQX did not result in any sensorimotor impairment apparent during observation of the rats' behaviour. Neither D-AP5 nor CNQX

appeared to affect motivational or sensorimotor processes underlying digging. Analysis of the overall dig time during the 60s retrieval phase of the probe trials did not reveal a difference between D-AP5 (19.00±1.67s) and aCSF (19.50±1.23s) infusion, regardless of the infusion time point (ANOVA; main effect of drug and interaction drug by time point, $F_{(1,13)}<1$) or between CNQX (12.82±1.51s) and aCSF (15.43±2.04s) infusion ($t_{(13)}=1.1$; $p>0.29$).

Blockade of AMPA, but not NMDA, receptor activation disrupts transmission at perforant path synapses in vivo (Expt. 7)

Transmission at perforant-path synapses onto dentate granule cells was markedly reduced after hippocampal CNQX infusion but relatively unaffected by infusion of D-AP5 (Fig. 3.5a). The absolute values of the baseline EPSP slopes before infusion did not differ across infusion groups (aCSF, 4.33±0.45mV/ms; D-AP5, 4.39±0.51mV/ms; CNQX, 5.11±1.31mV/ms; $F_{(2,16)}<1$). ANOVA of the normalized EPSP slopes after infusion revealed main effects of the infusion group ($F_{(2,16)}=7.9$; $p<0.005$) and 5min blocks ($F_{(32,512)}=27.5$; $p<0.0001$), as well as an interaction of both factors ($F_{(64,512)}= 12.6$; $p<0.0001$). The interaction reflected the temporary reduction of the EPSP slope in the CNQX groups compared with the D-AP5 and aCSF groups. Separate ANOVA of EPSP slopes 15–20min after infusion (Fig. 3.5a, gray bar), approximately corresponding to the time during which the encoding or retrieval phase took place in the behavioural paradigm of experiment 6, revealed a group effect ($F_{(2,16)}=18.2$; $p<0.0001$). *Post hoc* tests showed that the EPSP slope was lower in the CNQX group (46.40±9.32% of baseline) than in both the aCSF (100.66±1.93%; $p<0.0001$) and D-AP5 (91.01±4.87%; $p<0.0005$) groups, which did not differ ($p>0.35$).

Induction, but not maintenance, of in vivo long-term potentiation in dentate gyrus synapses depends on NMDA receptor activation (Expt. 7)

Hippocampal D-AP5 infusion completely blocked the induction of LTP without affecting its maintenance or expression (Fig. 3.5b). The absolute values of the baseline EPSP slopes did not differ across infusion groups (ACSF, 5.86±1.25mV/ms; AP-5, 6.04±1.05mV/ms; post-AP-5, 4.85±0.70mV/ms; $F_{(2,16)}$<1). ANOVA of the normalized EPSP slopes after infusion revealed main effects of the infusion group ($F_{(2,16)}$=5.3; p<0.02) and 5min blocks ($F_{(37,592)}$= 30.2; p<0.0001), as well as an interaction of both factors ($F_{(74,512)}$= 3.4; p<0.0001). This mainly reflected that, after tetanization, the EPSP slope in the ACSF and the post-AP-5 group showed pronounced LTP, whereas in the AP-5 group receiving infusion before tetanization, there was only a small post-tetanic potentiation lasting ~5 min. A separate ANOVA of EPSP slopes 20–25min after tetanization (Fig. 3.5b; gray bar), corresponding to the 20–25min that passed after the encoding phase in experiment 6 until the rats had to use the place memory in the retrieval phase, revealed a group effect ($F_{(2,16)}$= 9.3; p<0.0025). *Post hoc* comparisons demonstrated that EPSP slopes were markedly potentiated in the aCSF (123.62±2.89%; p<0.01) and post-D-AP5 (129.34±5.42%; p<0.001) groups compared with the D-AP5 group (103.87±3.88%).

Histology (Expt.6-7)

The tips of the infusion cannulae were located within the posterior dorsal hippocampi in experiments 6 and 7. In Expt. 7, all recording sites were located in the hilar region of the dentate gyrus; either just below the upper or just above the lower granule cell layer of the dentate gyrus, and the stimulation sites in the angular bundle (Fig. 3.6).

Figure 3.4: Dependence of encoding and retrieval of one-trial place memory on glutamate receptor activation in the hippocampus (Expt. 6)

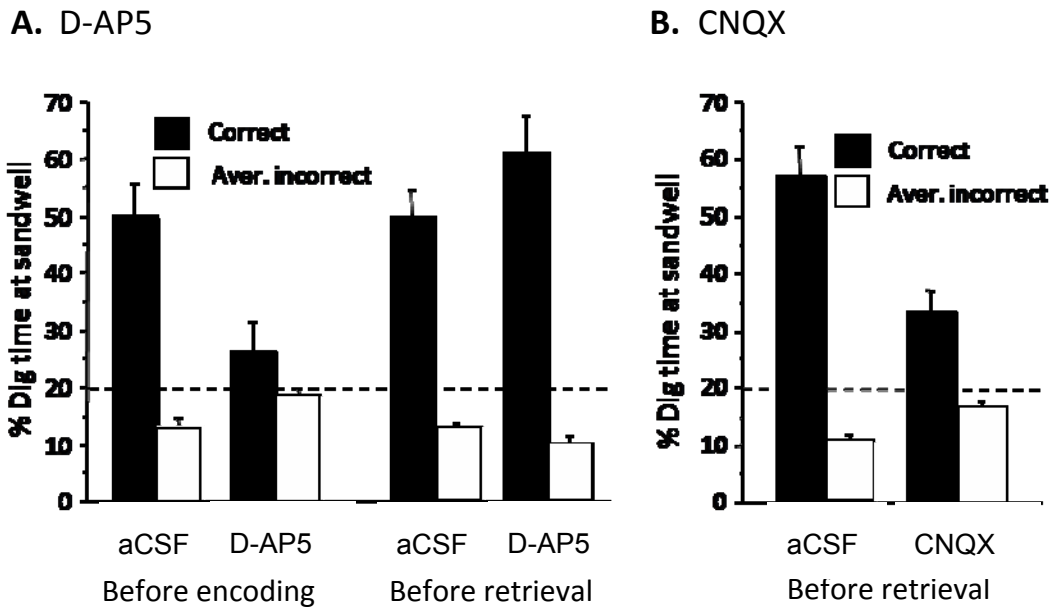


Figure 3.4. Dependence of encoding and retrieval of one-trial place memory on glutamate receptor activation in the hippocampus (Expt. 6). The percentage dig time at correct and novel sandwells (mean±1SEM) in probe trials is presented as measure of performance; stippled horizontal lines indicate chance (n=14). ***A)*** Performance when the NMDA receptor antagonist D-AP5 (30 mM, 1µl) or aCSF (1µl) was infused into the hippocampus 15min before the encoding or retrieval phase. ***B)*** Performance when the AMPA receptor antagonist CNQX (3mM, 1µl) or aCSF (1µl) was infused 15min before the retrieval phase.

Figure 3.5: Contribution of glutamate receptor activation for synaptic transmission and long-term potentiation in the hippocampus *in vivo* (Expt.7)

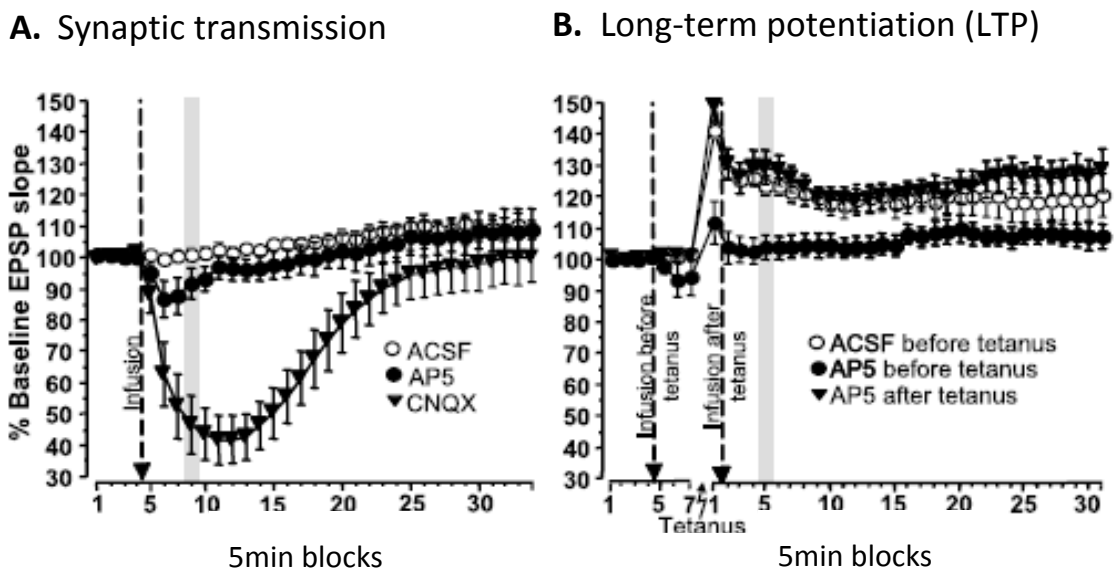


Figure 3.5. Contribution of glutamate receptor activation for synaptic transmission and long-term potentiation in the hippocampus *in vivo* (Expt. 7). EPSPs in the dentate gyrus evoked by low-frequency stimulation of the perforant path were recorded in anesthetized rats. Data are presented in 5min blocks as a percentage of the average EPSP slope during the 20min baseline recordings preceding the first infusion (percentage of baseline EPSP slope; mean±1SEM). aCSF (1µl), D-AP5 (30 mM, 1µl), or CNQX (3mM, 1µl) were infused at the times indicated by the arrows. ***A)*** Effects of hippocampal aCSF, D-AP5, or CNQX infusions on synaptic transmission. The vertical gray bar indicates 15–20min after infusion, corresponding to the time during which the encoding or retrieval phase took place after the hippocampal infusions in experiment 6. ***B)*** Effects of hippocampal aCSF or D-AP5 infusions on induction or maintenance of long-term potentiation. The gray bar indicates 20–25min after tetanization, corresponding to the delay between encoding and retrieval in experiment 6 (n=6-7 rats per group).

Figure 3.6: Histology (Expt. 6-7)

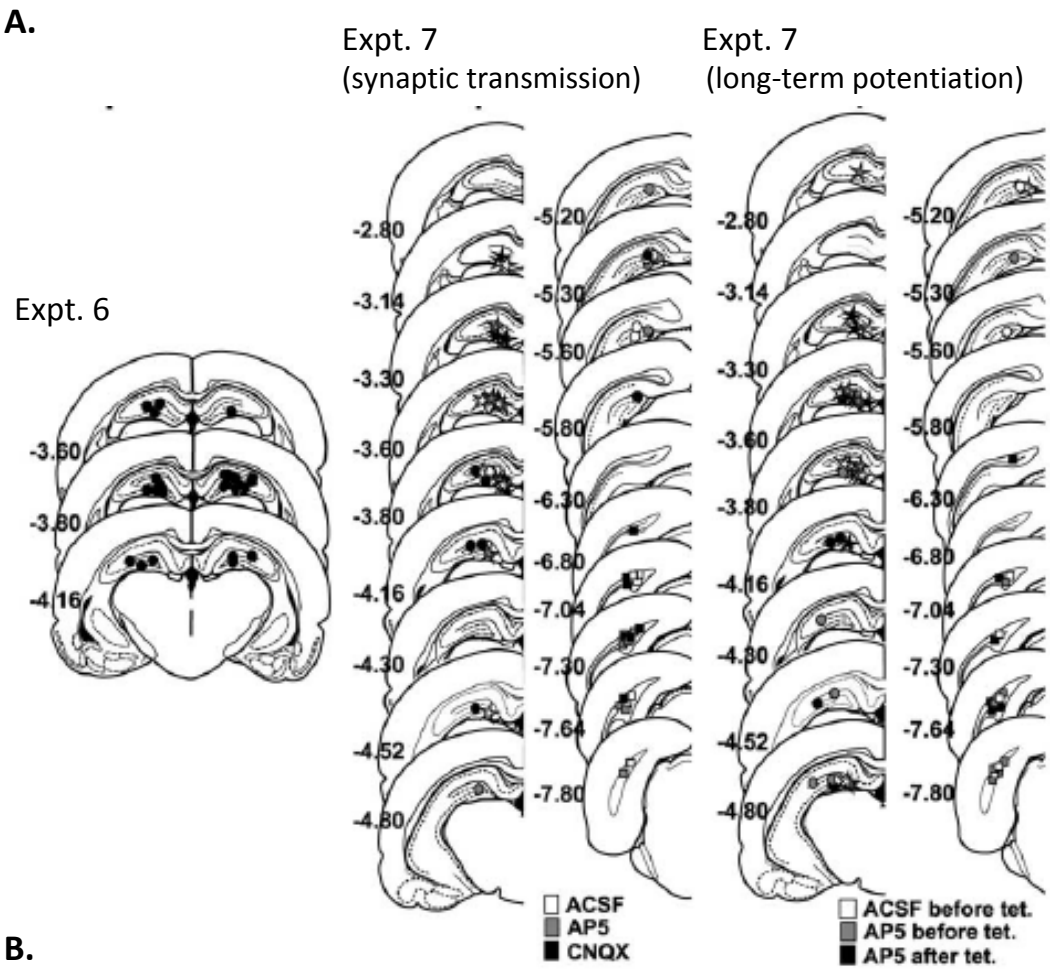


Figure 3.6. Infusion and electrode placements in the hippocampus (Expt. 6-7). ***A)*** Expt. 6: Approximate locations of infusion cannula tips (black dots) in both hemispheres. Expt. 7 (a-b): Approximate locations of the tips of infusion cannulas (dots), stimulation electrodes (squares), and recording electrodes (stars) in the left hemisphere, depicted for the different groups (gray-scale coding). Coronal sections are adapted from Paxinos and Watson (1998); the numbers indicate the distance from bregma in millimeters. ***B)*** Photographs of cresyl violet-stained sections showing, from left to right, representative recording, infusion, and stimulation sites. Recording and stimulation sites were marked by an electrolytic lesion. White arrowheads indicate the approximate locations of the tips of the infusion cannula and the electrodes (note: bipolar stimulating electrode).

3.2. Studies of place memory persistence in a modified version of the delayed matching-to-place task in the watermaze

3.2.1. Persistence of one-trial place memory (Expt. 8-10)

Pretraining

Stable asymptotic levels of performance are usually attained in the watermaze DMP task after 4 to 5 days of pretraining, a critical period during which animals learn the procedural requirements of the task (e.g. Steele and Morris, 1999). Rats used in experiments 8-10 were pretrained for 8 days. The 4 initial days comprised an inter-trial interval of 15s between all trials. From day 5 to day 8 the inter-trial interval between trials 1 and 2 was varied to familiarize the animals with the retention intervals tested in experiment 8, namely 15s, 1, 3 and 6h. Analysis of escape latencies obtained from day 1 to 4 showed a highly significant improvement with days ($F_{(3,57)}$=20.9; p<0.0001) and trials ($F_{(3,57)}$=38.5; p<0.0001, where trials corresponds to trials 1-4 of each day, see Fig. 3.7). One-trial learning, revealed as a significant decrease in escape latencies from trial 1 to trial 2, was observed from day 1 to day 4 of pretraining ($t_{(19)}$=2.9; p<0.01). The introduction of different retention intervals on day 5 did not allow analysing overall changes in performance throughout the 8 days of pretraining based on trial 2 escape latencies. However, the separate analysis of escape latencies for trials 3 and 4 showed that asymptotic performance was reached by days 5 to 6. The analysis of variance of average T3 and T4 latencies revealed a highly significant effect of day ($F_{(7,133)}$=21.3; p<0.0001), and latencies for these trials stabilized around 15s after day 5. Asymptotic levels of performance, with T3 and T4 latencies below 15s, were kept throughout pretraining days preceding probe days in these experiments.

Retention intervals of 15s, 1, 3 and 6h (Expt. 8)

One-trial place memory strength declined with increasing retention intervals, as observed in the event arena task (see Expt. 5). Again, decay of memory was mostly evident by analysis of persistent search during probe trials. Analysis of the percentage of time spent swimming in the correct zone (zone analysis) revealed a highly significant effect of retention interval on memory strength ($F_{(3,57)}$=9.5; p<0.0001) (see Fig. 3.8a above). *Post hoc* Fisher's PLSD tests showed a significant difference between 15s and all other retention intervals (p<0.0005); no significant differences were found between retention intervals of 1, 3 and 6h. Above chance performance was observed with all retention intervals ($t_{(19)}$=4.3; p<0.0005). Decay of memory strength with increasing retention intervals was also suggested by the analysis of crossing latencies during probe trials, which increased from an average of 21.3±4s at 15s to an average of 35.8±6s at 6h, however, this failed to reach significance ($F_{(3,57)}$=1.2; p=0.33), as well as the analysis of latency savings between trials 1 and 2 ($F_{(3,57)}$=0.2) (see Fig 3.8a below).

Retention intervals of 15s, 15min, 30min and 1h (Expt. 9)

This experiment investigated the marked decay of memory observed within the first hour of acquisition in experiment 8. For this purpose memory was further tested after retention intervals of 15 and 30min. Again, the overall ANOVA of the percentage of time spent in the correct zone showed a significant effect of retention interval on memory strength ($F_{(3,57)}$=3.4; p<0.05) (see Fig 3.8b above). *Post-hoc* Fisher's PLSD tests revealed a significant difference between retention intervals of 15s and of 15min or 1h (p<0.05; p<0.005). Above chance performance was obtained with all retention intervals ($t_{(19)}$=5,1; p<0.0001). Overall, crossing latencies increased with longer intervals, but this failed to reach significance ($F_{(3,57)}$=2.4; p=0.08) (see Fig 3.8b below). Analysis of latency savings between trials 1 and 2 revealed a main effect of retention interval ($F_{(3,57)}$=3.3; p<0.05), however, this did not reflect a decline of

memory strength with increasing retention intervals; mean savings were of 32.5±8s for 15s, 12±7s for 15min, 44.3±6s for 30min and 18.4±9s for 1h.

Retention intervals of 6 and 24h (Expt. 10)

This experiment tested the persistence of memory beyond 6h. Zone analysis, crossing latencies, or latency savings, did not reveal an effect of retention interval on performance ($F_{(1,19)}$=0.4; $F_{(1,19)}$=0.1; $F_{(1,19)}$=0.2; respectively) (see Fig 3.8c). However, planned paired Student's *t* test comparisons showed that performance was above chance after 6h ($t_{(19)}$=2.6; p<0.05) but not 24h ($t_{(19)}$=1.5; p=0.14).

Overall analysis

The results from experiments 8 to 10 suggest that there is a gradual forgetting of one-trial place memory within 24h of acquisition. They also suggest that the used measures of performance have different sensitivities to variations in memory strength. While zone analysis clearly revealed a decline of memory within 24h (Fig. 3.8d, left), crossing latencies, and mostly, latency savings, were more resistant to the effect of increasing retention intervals (Fig. 3.8d right). Finally, it is worth noting the high reliability of the performance levels obtained with retention intervals tested repeatedly across experiments (15s, 1h and 6h). Most notably, the direct comparison of the time spent swimming in the correct zone at these retention intervals did not reflect a difference between experiments [Expt. 1-2: 15s RI ($t_{(19)}$<1), 1h RI ($t_{(19)}$<1); Expt. 1-3: 6h RI ($t_{(19)}$<1)]. Based on the results of experiments 8-10, subsequent experiments will be analysed focusing mainly on the measure of persistent search during probe trials.

Figure 3.7: Acquisition of the delayed matching-to-place task in the watermaze and performance during inter-probe days (Expt. 8-10)

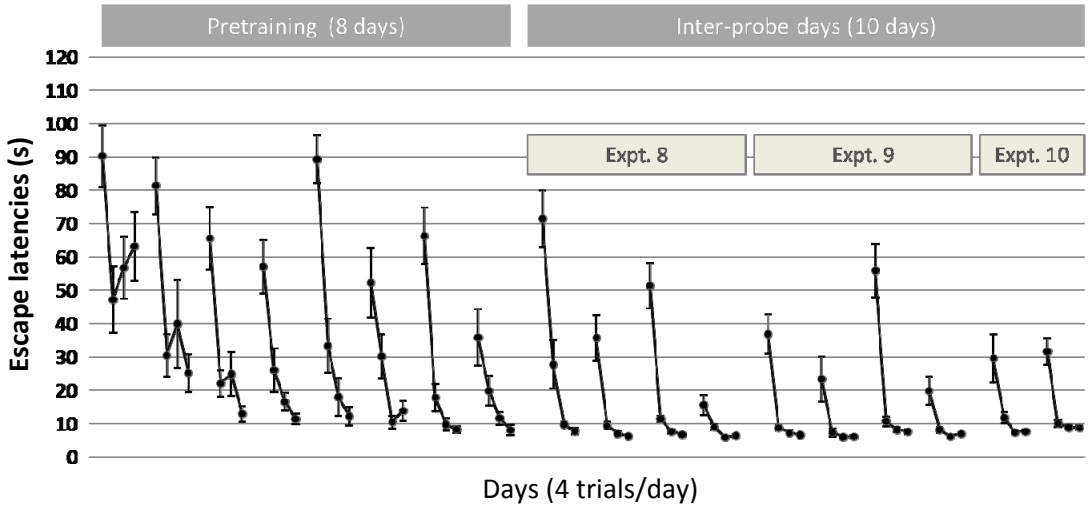


Figure 3.7. Acquisition of the delayed matching-to-place task in the watermaze and performance during inter-probe days (Expt. 8-10). During pretraining rats (*n*=20) were given 4 standard trials (T1-T4) per day, over 8 days. From day 1 to day 4 the ITI between trials 1 and 2 was 15s. From day 5 to day 8, the interval was varied in order to familiarize the animals with the retention intervals tested in experiment 8 (i.e. 15s, 1, 3 and 6h). Intervals between trials 2 and 4 were kept constant at 15s throughout pretraining and inter-probe days. During inter-probe days the interval between trials 1 and 2 was either matched with the retention intervals tested in subsequent probe days (Expt. 8 and 9) or fixed at 15s (Expt. 10). The averaged performance for the different groups of animals tested with different T1-T2 intervals during pretraining and inter-probe days is shown. Mean±1SEM.

Figure 3.8: Persistence of one-trial place memory in the modified delayed matching-to-place task in the watermaze (Expt. 8-10)

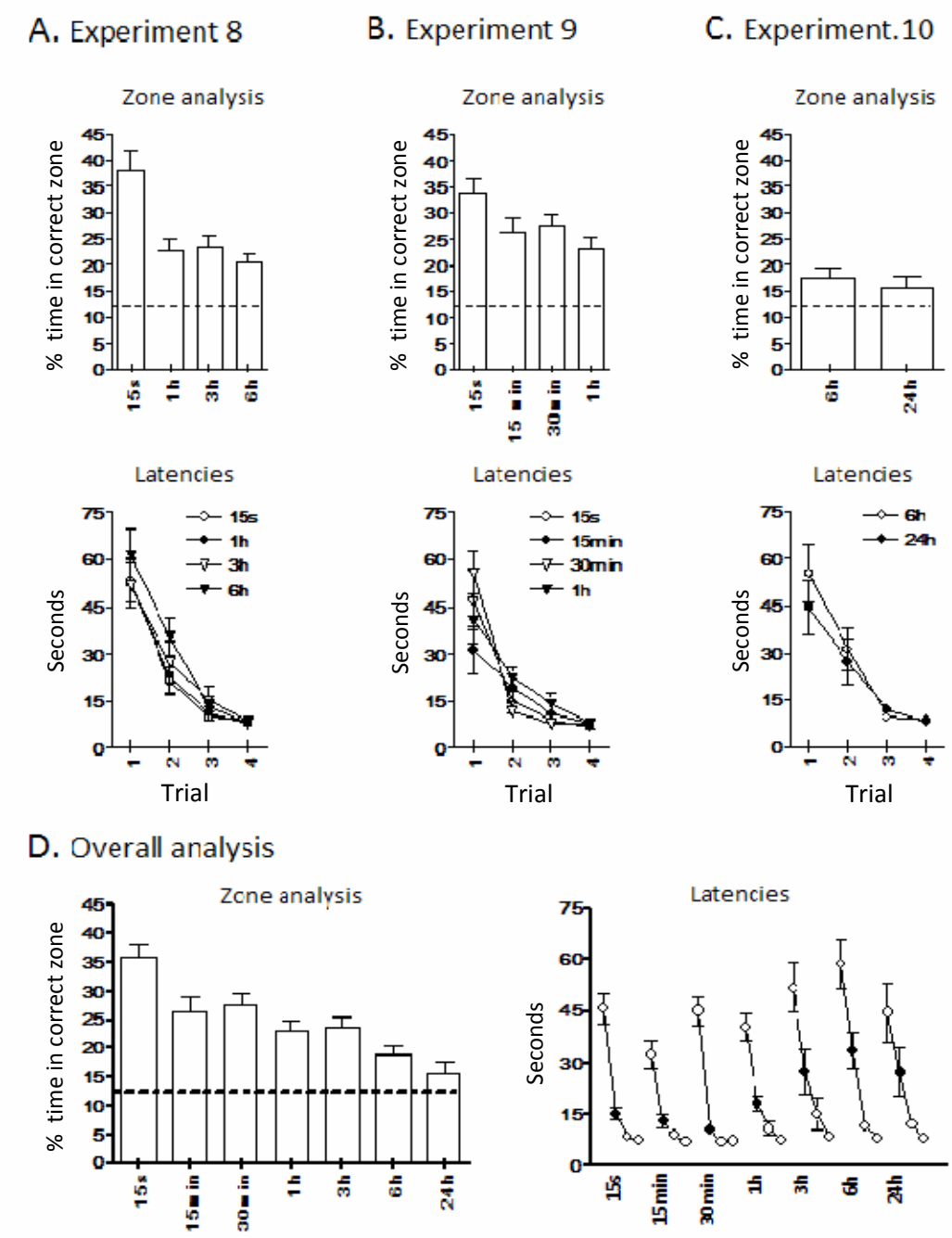


Figure 3.8. *Expt. 8-10: Persistence of one-trial memory in the watermaze*. ***A-C)*** Zone analysis and probe day latencies are shown for experiments 8 *(A)*, 9 *(B)* and 10 *(C).* Expt. 8 tested retention intervals of 15s, 1, 3 and 6h; expt. 9 tested retention intervals of 15s, 15min, 30min and 1h; and expt. 10 tested retention intervals of 6 and 24h. Retention intervals of 15s, 1h and 6h were tested repeatedly across experiments. ***D)*** Overall analysis. Performance at 15s, 1 and 6h is averaged across experiments. *Left:* Percentage of time spent in correct zone (probe trials). *Right*: Probe day latencies. Stippled horizontal lines indicate chance value for % time in correct zone. Filled circles in D *(right)* represent probe trial crossing latencies. Mean±1SEM (n=20).

3.2.2. Enhancement of long-term place memory strength by repetition and spacing of acquisition trials (Expt. 11-13)

Pretraining

Rats used in experiments 11-13 showed a similar task acquisition to animals used in experiments 8-10; asymptotic levels of performance, with T3 and T4 latencies below 15s, were observed from day 5-6 of pretraining and were kept throughout inter-probe days (*data not shown*). In experiment 11, the strength of 6h memory for a single acquisition trial (30s on platform) did not differ between days 7 and 10 of pretraining (zone analysis; $t_{(17)}<1$). The average percentage values of swimming time in the correct zone obtained on these days, 17.5±2.2 and 16.1±1.4 respectively, were comparable to those obtained subsequently on probe days (16.9±1.8; see Fig. 3.9b, 1T-30s).

Experiment 11

Increasing the number and/or the temporal distribution of encoding trials enhanced long-term place memory. Analysis of the percentage of time that rats spent searching the correct zone during probe trials revealed a main effect of encoding conditions on performance ($F_{(5.85)}=3.6$; $p<0.01$; Fig. 3.9b). Further analysis with *post-hoc* Fisher's PLSD tests revealed that memory for a single encoding trial (30s condition) was weaker than performance obtained with spaced ($p<0.05$), but not massed ($p=0.05$), multi-trial conditions. Six massed trials ($p<0.05$) or 3 spaced trials ($p<0.005$) produced stronger memory than 3 massed trials. Interestingly, memory strength for 3 or 6 spaced trials did not differ ($p=0.69$), which may imply that performance may have reached ceiling levels with 3 spaced trials. Finally, no difference was found between conditions where the rats were given a single acquisition trial and allowed to spend 6 or 30s on the platform ($p=0.4$). Performance was above chance for all encoding conditions ($t_{(17)}=2.4$; $p<0.05$).

While zone analysis revealed a clear effect of both repetition and temporal distribution of encoding trials on memory strength, crossing latencies were only sensitive to the effect of trial repetition on performance (see *Supplement 3a*).

Experiments 12 and 13

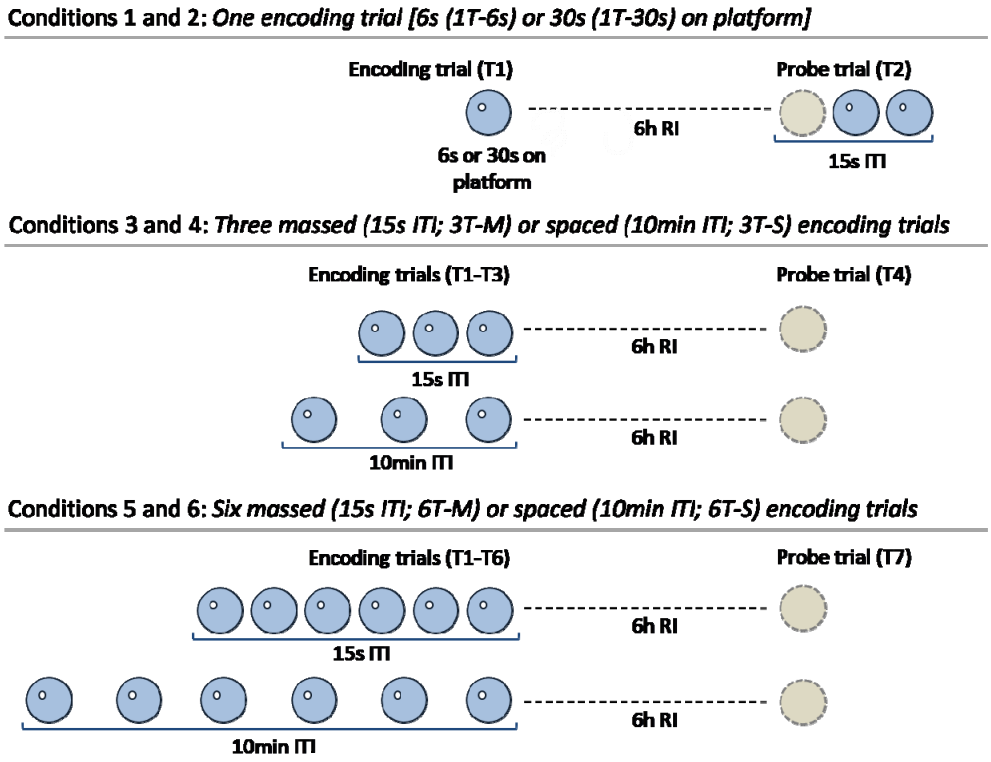
As predicted by previous results (Expt. 11), memory for three spaced swim trials (10min ITI) was stronger than memory for a single encoding trial after retention intervals of 6h (Expt. 12) and 24h (Expt. 13). Zone analysis revealed a clear enhancement of memory strength with 3 spaced trials ($F_{(1.15)}$=8.4; p<0.05) and above chance performance for both encoding conditions ($t_{(15)}$<0.005; see Fig. 3.9c). Neither the analysis of crossing latencies, nor analysis of latency savings, showed a significant difference between one-trial memory or memory for 3 spaced encoding trials after 6h ($F_{(1.15)}$=0.97 and $F_{(1.15)}$=0.56, respectively; see *Supplement 3b*). The same pattern of results was observed in Expt. 13, when memory was assessed 24 later. Zone analysis revealed a difference between encoding conditions ($F_{(1.15)}$=6.7; p<0.05; Fig. 3.9d), which was not detected by analysis of crossing latencies ($F_{(1.15)}$=3.9; p=0.07) or latency savings ($F_{(1.15)}$=0.26; see *Supplement 3c*), and above chance performance was observed for both encoding conditions ($t_{(15)}$<0.005).

Summary

Overall, the results from experiments 8-13 show that one-trial allocentric place memory decays rapidly within minutes to hours of acquisition, with weak memory consistently detectable 6h, but not 24h, after acquisition. Repetition and spacing of acquisition trials were shown to enhance the strength of long-term (≥ 6h) place memory within a single training session. Importantly, the use of search preference as measure of performance in the modified DMP task enhanced the sensitivity of the task to variations in place memory strength.

Figure 3.9: Enhancement of long-term place memory strength by repetition and spacing of acquisition trials (Expt. 11-13)

A. Expt. 11 (encoding conditions)



B. Expt. 11 (zone analysis) **C.** Expt. 12 (6h RI) **D.** Expt. 13 (24h RI)

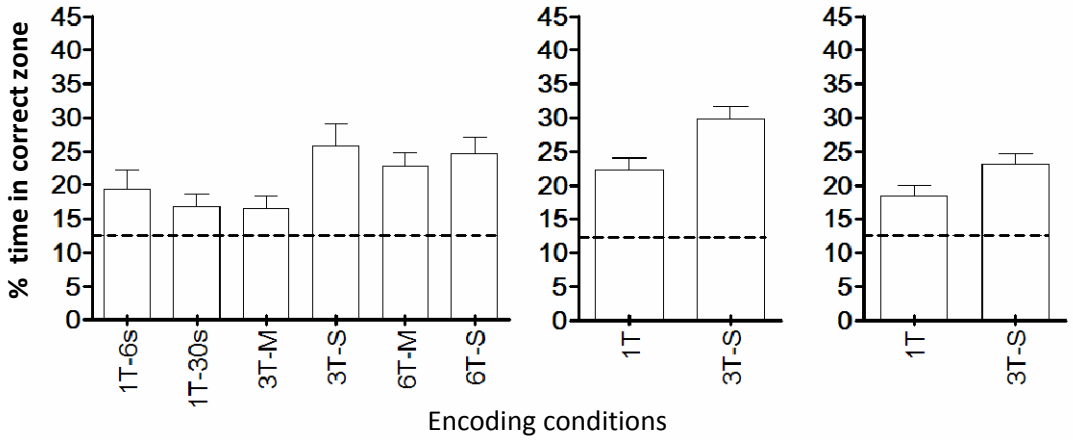


Figure 3.9. Enhancement of long-term place memory strength by repetition and spacing of acquisition trials (Expt. 11-13). ***A)*** Expt. 11 assessed long-term memory [6h retention interval (RI)] strength for the following types of encoding event: 1 encoding trial with either 6s (1T-6s) or 30s (1T-30s) on platform; 3 encoding trials with 15s (massed; 3T-M) or 10min (spaced; 3T-S) inter-trial intervals; and finally, 6 encoding trials with 15s (massed; 6T-M) or 10min (spaced; 6T-S) inter-trial intervals (n=18). ***B)*** Percentage of time in correct zone during probe trials is shown for Expt. 11. ***C-D)*** Memory strength for a single encoding trial or 3 spaced encoding trials (10min ITI; 3T-S) was further investigated in a new batch of sixteen animals 6h (Expt. 12) or 24h (Expt. 13) after encoding. Stippled horizontal lines indicate chance value for % swim time in correct zone. Mean±1SEM.

3.3. Investigating the implications of the STC hypothesis for the formation of long-term place memory in the watermaze

3.3.1. Characterizing "weak" and "strong" encoding events (Expt. 14)

Prior to this experiment, 3 spaced "swim" trials, in which rats were allowed 2min to swim to the platform and 30sec on the platform, were shown to produce stronger memory than a single acquisition trial (Expt. 11-13); however, memory for both types of encoding event could be detected after long-term retention intervals. This experiment established 3 spaced "swim" trials as a "strong" encoding event leading to the formation of strong short-term memory (30min; Cond. 1) and detectable long-term memory (24h; Cond. 2), and 3 spaced "placement" trials, in which rats were simply placed on the platform for 30s, as a "weak" encoding event leading to the formation of weak memory detectable 30min (Cond. 3), but not 24h (Cond. 4), after acquisition. Probe trials revealed a clear effect of trial type, and retention interval, on memory strength (see Fig. 3.10b). Rats spent more time searching the correct zone when they were given "swim" trials than when they were given placement trials ($F_{(1.15)}$=17.4; p<0.001; no interaction was observed between trial type and retention interval: $F_{(1.15)}$=3.4; p=0.087) and overall, memory for both types of encoding event was stronger at 30min than at 24h ($F_{(1.15)}$=17.9; p<0.001). From 30min to 24h the time rats spent searching the correct zone decreased about 15% after "swim" trials (similar decline was observed with a single encoding trial – see Fig. 3.8) and 8% after placement trials. Above chance performance was observed for "swim" trials at both retention intervals ($t_{(15)}$=3.2; p<0.01), however, memory for placement trials, detectable at 30min ($t_{(15)}$=2.3; p<0.05), could not be detected after 24h ($t_{(15)}$<1). Stronger memory for "swim" trials was also indicated by lower crossing latencies during probe trials even though this difference failed to reach significance ($F_{(1.15)}$=4.3; p=0.055). Latencies failed to reveal a main effect of retention interval on performance ($F_{(1.15)}$=1.5; p=0.24) (*Suppl 4*).

Figure 3.10: Encoding events inducing the formation of either short- or long-term place memory in the watermaze (Expt. 14)

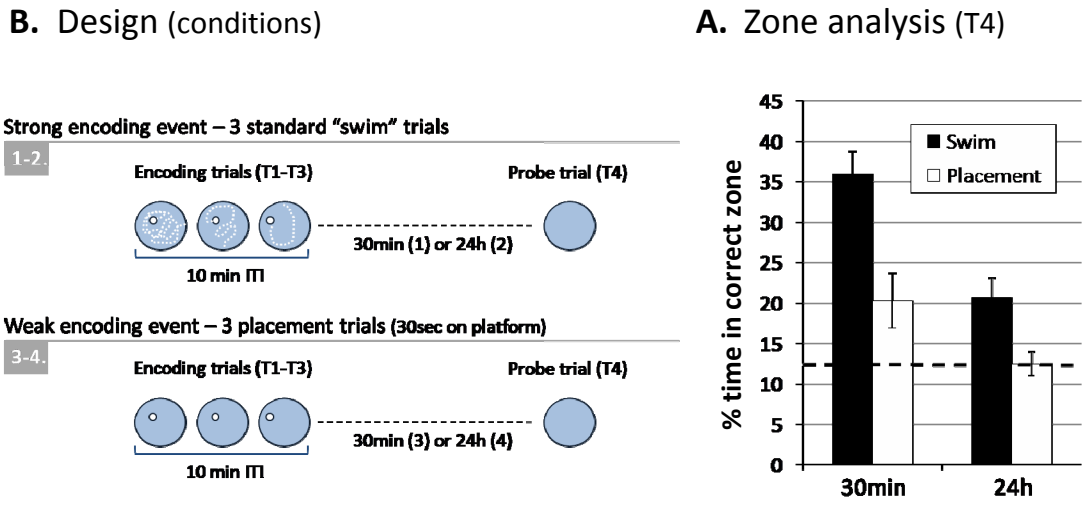


Figure 3.10. *Encoding events inducing the formation of either short- or long-term place memory in the watermaze (Expt. 14).* ***A)*** Rats (n=16) were given 3 "swim" trials (Cond. 1-2) or 3 placement trials (Cond. 3-4). Encoding trials were separated by 10min (spaced training). During a "swim" trial rats were allowed 2min to swim and find the platform and 30s on the platform. During a placement trial rats were only placed on the platform for 30s. Retention of memory for both "swim" and placement trials was tested 30min (Cond. 1 and 3) or 24h (Cond. 2 and 4) after acquisition. ***B)*** Percentage of time rats spent searching the correct zone during probe trials. Stippled horizontal lines indicate chance level. Mean±1SEM.

3.3.2. Investigating behavioural analogues of the "strong-before-weak" and "weak-before-strong" paradigms in the watermaze (Expt. 15-17)

Task acquisition and performance during inter-probe days (Expt. 15-17)

Rats rapidly acquired the task (see Fig. 3.11). Significant T1-T2 latency savings were observed from the first day of pretraining and T3-T4 escape latencies reached asymptotic levels of about 15s approximately by days 6-7. Importantly, the rate of task acquisition observed in this and subsequent experiments, in which rats were pretrained in two watermazes simultaneously, did not differ from that observed in experiments in which rats were pretrained in a single watermaze [e.g. compare pretraining between this experiment and Expt. 8-10 (Fig. 3.7); also, compare Expt. 18 and Expts. 20/21 (*Supplements 8a and 10/11a*) which followed the same exact pretraining design (with start positions and platform positions counterbalanced) but used one (Expt. 18) or two (Expt. 20/21) watermazes].

Escape latencies during inter-probe days (4 trials/day; 15s T1-T4 ITI) were stable and at an asymptotic level throughout the experiments (see Fig. 3.11). The variability of T1 escape latencies, presumably due to the use of different platform positions each day, was homogeneous across experiments. In addition, T3-T4 escape latencies remained at asymptotic levels. Training during inter-probe days was carried out in a single watermaze (upstairs watermaze) for practical reasons. It is not possible to compare performance between watermazes directly in these experiments because animals were tested with different retention intervals in each watermaze [mostly 24h in the "upstairs" watermaze and 26h in the "downstairs" watermaze; *see below*]. However, the stability of performance obtained during probe trials in the upstairs watermaze for conditions tested repeatedly across experiments suggests that overtraining did not increase memory strength for encoding events occurring in this watermaze (*see below*).

Figure 3.11: Task acquisition and performance during inter-probe days (Expt. 15-17)

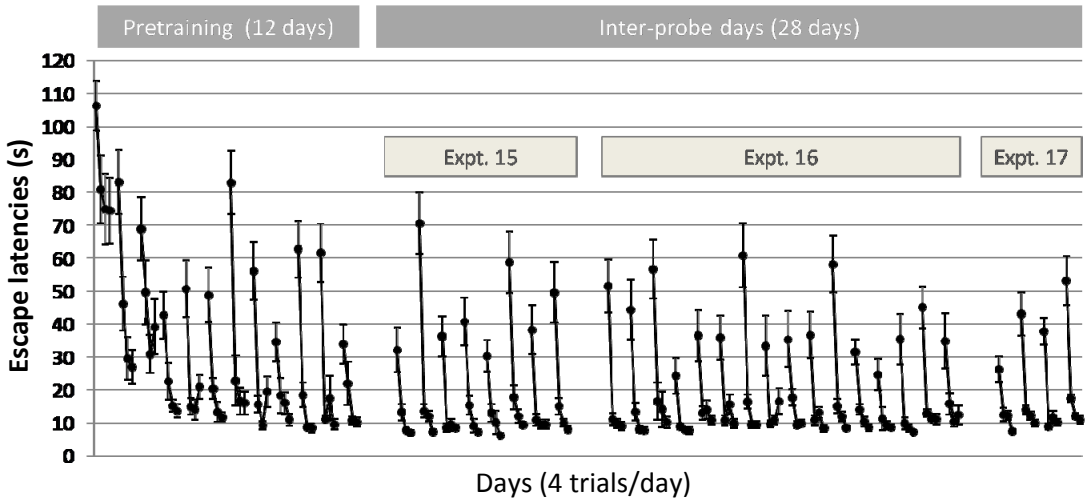


Figure 3.11. *Task acquisition and performance during inter-probe days (Expt. 15-17).* Rats (n=20) were given 4 trials a day (15s ITI) to a platform position that changed between, but not within days. During pretraining rats were trained in two different watermazes. During inter-probe days rats were only trained in one watermaze. Two inter-probe days preceded each pair of probe days in these experiments. Black lines connect the averaged latencies for trials 1-4 of each day. Mean±1SEM.

In a different experiment the direct comparison of performance between watermazes immediately after pretraining revealed similar levels of performance for the same encoding conditions (see Expt. 21 below).

Experiment 15

Weak encoding events (3 spaced placement trials) did not produce memory detectable at 24h when preceded (Cond.1; *strong-before-weak paradigm*), or followed (Cond. 3; *weak-before-strong paradigm*), 50min apart, by strong "modulatory" events occurring in a different environment. Also, replacement of strong "modulatory" events by weak "control" events in the strong-before-weak and weak-before-strong paradigms (Cond. 2 and 4, respectively) did not modify the strength of memory for weak "target" events. Long-term memory (26h retention interval) was always detected for strong "modulatory" events and never detected for weak "control" events.

Performance for weak (target) encoding events in the upstairs watermaze (24h retention interval): Neither zone analysis ($F_{(1.19)}$<1; see Fig. 3.12b left; *black*) nor analysis of crossing latencies ($F_{(1.19)}$=2.4; p=0.14; see *Supplement 5 left; black*), revealed an interaction between "modulatory" and "target" encoding events, as no difference in performance was found between test (1 and 3) and control (2 and 4) conditions. Performance for weak "target" events did not differ between paradigms ($F_{(1.19)}$<1 for zone analysis and crossing latencies). Although above chance performance was detected for the test condition in the strong-before-weak paradigm ($t_{(19)}$=2.5; p<0.05), the percentage of time rats spent swimming in the correct zone did not differ between test (Cond. 1) and control (Cond. 2) conditions in this paradigm ($t_{(19)}$<1). Zone analysis revealed chance levels of performance for conditions 2-4 ($t_{(19)}$=1.4; p=0.2).

Performance for strong (modulatory) and weak (control) encoding events in the downstairs watermaze (26h retention interval): As expected, only strong encoding events produced long-lasting memory detectable 26h later (Cond. 1 and 3) and performance for both types of encoding

event did not differ between paradigms. Both zone analysis (see Fig. 3.12b left; *red*) and analysis of crossing latencies (see *Supplement 5 left; red*) revealed a main effect of type of encoding [$F_{(1.19)}$=36.7; p<0.0001 and $F_{(1.19)}$=8.2; p<0.01, respectively), but no effect of paradigm ($F_{(1.19)}$<1 and $F_{(1.19)}$=1.4; p=0.26, respectively), on probe trial performance. Also, no interaction was observed between type of encoding and type of paradigm (zone analysis: $F_{(1.19)}$=3.4; p=0.08; crossing latencies: $F_{(1.19)}$<1). Finally, above chance performance was detected for strong ($t_{(19)}$=5.2; p<0.0001), but not weak ($t_{(19)}$<1), encoding events (see Fig. 3.12b left; *red*); crossing latencies were also lower after strong encoding events in both paradigms (see *Supplement 5 left; red*).

Overall analysis: The absence of a synergistic interaction between strong "modulatory" events and weak "target" events was further confirmed by the lack of a statistically significant correlation between performance values obtained in both watermazes either in the "strong-before-weak" [*zone analysis*: $r_{(19)}$=0.34; p=0.07; *one-tailed* (Fig.3.12b, right; *black line*); crossing *latencies*: $r_{(19)}$=0.02; p=0.46; *one-tailed* see *Supplement 5 right; black line*)], or the "weak-before-strong" [*zone analysis*: $r_{(19)}$=0.17; p=0.23; *one-tailed* (Fig.3.12c, right; *grey line*); *crossing latencies*: $r_{(19)}$=0.03; p=0.45; *one-tailed* (*Supplement 5 right; grey line*)], paradigms.

There are several possible explanations for the failure to find a synergistic interaction between "modulatory" and "target" encoding events. One such explanation is proactive and retroactive interference (e.g. Underwood, 1957). It is possible that the strong "modulatory" events and/or the ensuing memory worsened the formation and/or recall of memory for subsequent (proactive interference) or previous (retroactive interference) "target" events. In experiment 14, memory for weak encoding events was shown to persist over 30min but not 24h. The next experiment investigated if short-term memory for weak "target" events was still detectable in the "strong-before-weak" paradigm.

Experiment 16

Short-term memory (30min) for weak "target" events was detected in the "strong-before-weak" paradigm independently of the strong (Cond. 1), or weak (Cond. 3), nature of the preceding encoding events. Whether preceded by strong "modulatory" events (Cond. 2), or weak "control" events (Cond. 4), the target encoding events never produced memory detectable after a 24h retention interval. As predicted, long-term memory was always detected for "modulatory" events but not for weak "control" events.

The experiment was run in two replications and a main effect of series was revealed by analysis of crossing latencies ($F_{(1.19)}$=6.1; p<0.05). As further analysis of crossing latencies only showed a significant interaction between series and watermaze ($F_{(1.19)}$=13.2; p<0.005) but no interaction between series and condition ($F_{(1.19)}$=2.8; p>0.05), and zone analysis did not reveal a main effect of series ($F_{(1.19)}$=2.7; p=0.12), data from the two series were averaged for further analysis.

Performance for weak (target) encoding events in the upstairs watermaze (24h retention interval): Analysis of the time rats spent searching the correct zone during probe trials revealed that memory for weak "target" events was always detectable when assessed 30min ($t_{(19)}$=4.0; p<0.001) after encoding and, as seen in experiment 15, never detected after 24h ($t_{(19)}$=1.8; p=0.08; see Fig. 3.13b left; *black*). Zone analysis also revealed a significant interaction between retention interval and "downstairs" encoding type ($F_{(1.19)}$=6.9; p<0.05); however, this was due to the fact that performance at 24h for the weak "target" event was lower when this event was preceded by a strong "modulatory" event ($t_{(19)}$=2.2; p=0.043); no significant difference in performance was found between conditions testing memory after 30min ($t_{(19)}$=1.6; p=0.12). Analysis of crossing latencies confirmed that animals were faster to reach the platform position at 30min regardless of the nature of the preceding encoding event in the downstairs watermaze ($F_{(1.19)}$=23.8; p<0.0001). No main effect of "downstairs" encoding type or interaction were observed ($F_{(1.19)}$<1) (see *Supplement 6 left; black*).

Performance for strong (modulatory) and weak (control) encoding events in the downstairs watermaze (26h retention interval): Zone analysis revealed a highly significant effect of type of encoding event ($F_{(1.19)}$=37.0; p<0.0001), but no effect of retention interval in the upstairs watermaze ($F_{(1.19)}$=1.5; p=0.24), on performance during probe trials (see Fig 3.13b left; *red*). No interaction was observed between encoding event and upstairs retention interval ($F_{(1.19)}$=4.2; p>0.05). Above chance performance was observed for strong ($t_{(19)}$=4.0; p<0.0007) but not weak ($F_{(1.19)}$=1.4; p=0.17) encoding events. As in experiment 15, these results confirmed that only strong "modulatory" events produced memory detectable 26h after acquisition. Analysis of crossing latencies also revealed that rats took less time to reach the area occupied by the platform when given strong encoding events ($F_{(1.19)}$=23.8; p<0.0001) (*Supplement 6 left; red*); this occurred independently of the retention interval being tested in the upstairs watermaze ($F_{(1.19)}$<1).

Overall analysis: Further analysis of the time that rats spent searching the correct zone during probe trials did not reveal a significant correlation between performance obtained in both watermazes in the "strong-before-weak" conditions either at 30min or 24h retention intervals (Cond. 1: $r_{(19)}$= -0.03; p=0.45; Cond. 2: $r_{(19)}$=0.17; p=0.23; *one-tailed*) (Fig. 3.13b right; *black and gray bars*), which was also observed with crossing latencies (Cond. 1: $r_{(19)}$= -0.005; p=0.49; Cond 2. $r_{(19)}$=-0.07; p=0.37; *one-tailed*) (*Supplement 6 right; black and grey bars*). Although these results did not provide direct evidence that formation and/or retention of memory for "modulatory" and "control" encoding events did not interfere with the formation and/or retention of memory for "target" events (see Expt. 20), they did show that the nature of the preceding encoding events did not determine the strength of short-term memory for "target" encoding events. The absence of a synergistic interaction between "modulatory" and "target" encoding events in the "strong-before-weak" paradigm replicates the results from Expt. 15.

Experiment 17

Reducing the time interval between "modulatory" (Cond. 1) or "control" (Cond. 2) encoding events and "target" encoding events to 5 min did not result in the detection of long-term (24h) memory for "target" encoding events. As in experiment 15 and 16, "modulatory", but not "control", encoding events produced memory detectable 26h later.

Performance for "target" encoding events in the upstairs watermaze (24h retention interval): Neither zone analysis ($t_{(19)}$<1; Fig. 3.14b left; *black*), nor analysis of crossing latencies ($t_{(19)}$<1; *Supplement 7 left; black*), revealed a significant difference in performance between conditions. Performance was at chance levels in both conditions ($t_{(19)}$<1; Fig. 3.14b left; *black*)

Performance for "modulatory" and "control" encoding events in the downstairs watermaze (26h retention interval): As expected, zone analysis revealed a significant difference in performance between conditions ($t_{(19)}$=3.04; p<0.01); memory was detected for the strong "modulatory" event ($t_{(19)}$=4.9; p<0.0001), but not the weak "control" event ($t_{(19)}$=1.8; p=0.08; see Fig. 3.14b left; *red*). Although average crossing latencies were slightly higher when the animals were given "control" encoding events (Cond. 2), no significant statistical difference was observed between conditions ($t_{(19)}$=1.2; p=0.26; *Supplement 7 left; red*).

Overall analysis: The lack of a statistically significant correlation between performance values obtained during probe trials in the upstairs and downstairs watermazes in the "strong-before-weak" condition [*zone analysis*: $r_{(19)}$=-0.07; p=0.39; *one-tailed* (Fig.3.14b, right); crossing *latencies*: $r_{(19)}$=0.20; p=0.20; *one-tailed* (*Supplement 7 right*)] further supports the absence of a synergistic interaction between "modulatory" and "target" encoding events.

3.3.3. Dependence of long-term memory for "strong" encoding events on protein synthesis in the hippocampus (Expt. 18-19)

Dependence of long-term memory for "strong" encoding events on protein synthesis in the hippocampus (Expt. 18)

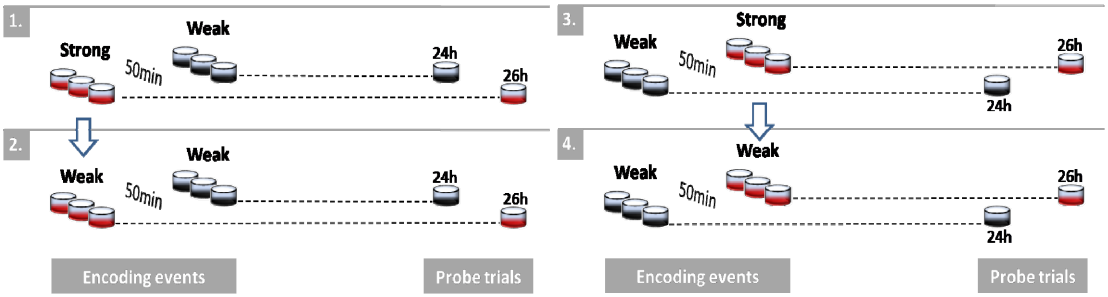
Bilateral intra-hippocampal infusions of the protein synthesis inhibitor anisomycin disrupted the formation of long-term memory for strong encoding events. Analysis of swim speed during the first encoding trial did not show a difference between drug and vehicle conditions ($t_{(11)}$<1; see Fig. 3.15b), rendering the analysis of latencies a valid measure of performance. Analysis of escape latencies obtained during the three encoding trials did not reveal a main effect of condition (i.e. aCSF or anisomycin; $F_{(1,11)}$<1) nor a significant interaction between condition and encoding trial ($F_{(2,22)}$<1; see Fig. 3.15c). However, in the probe trial 6h later, both the analysis of crossing latencies ($t_{(11)}$=2.7, p<0.05) and zone analysis ($t_{(11)}$=2.8, p<0.05; see Fig. 3.15d) revealed a strong impairment of memory strength after anisomycin infusions. Treated rats took longer to cross the zone occupied by the platform during probe trials (Fig. 3.15c; trial 4) and, unlike vehicle controls ($t_{(11)}$=3.9, p<0.005), showed no memory for its position ($t_{(11)}$<1; see Fig. 3.15d). Overall, these results suggest that formation of long-term memory for strong encoding events requires the synthesis of new proteins in the hippocampus.

Figure 3.12: Investigating behavioural analogues of the "strong-before-weak" and "weak-before-strong" paradigms in the watermaze (Expt. 15)

A. Design (conditions)

1-2. "Strong"-before-"weak" paradigm

3-4. "Weak"-before-"strong" paradigm



B. Zone analysis (probe trials)

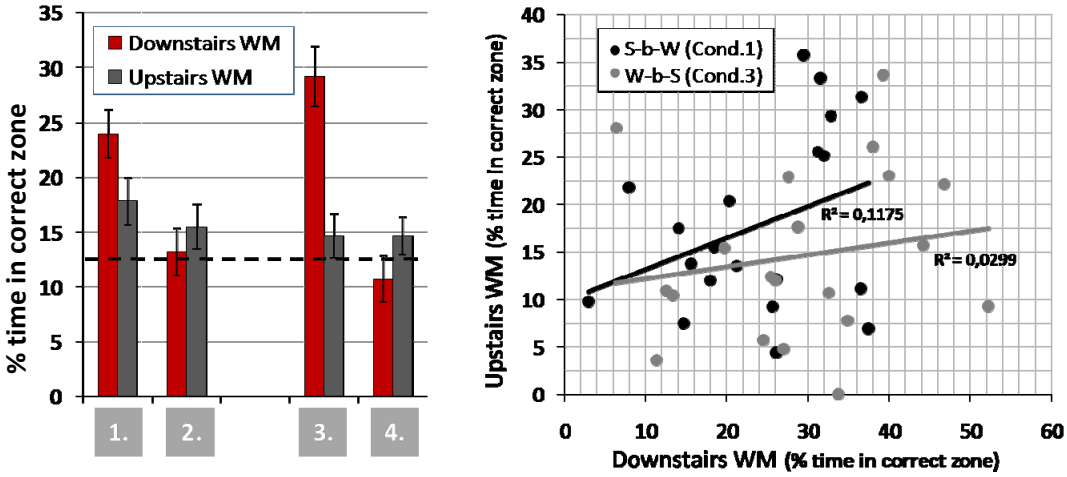
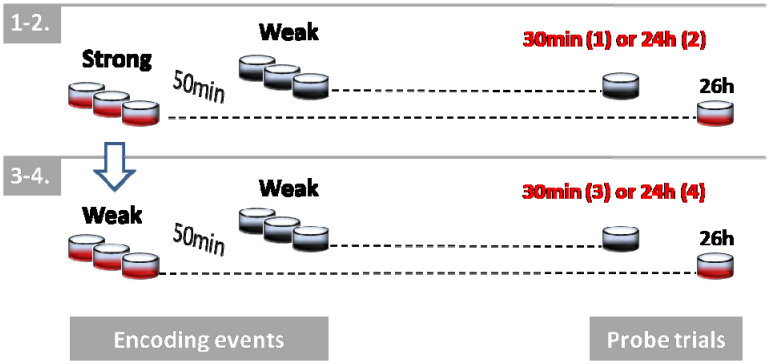


Figure 3.12. *Investigating behavioural analogues of the "strong-before-weak" and "weak-before-strong" paradigms in the watermaze (Expt. 15).* ***A)*** Strong-before-weak and weak-before-strong paradigms in the watermaze. Rats (n=20) were given a weak encoding event in the upstairs watermaze (black) preceded (Cond. 1), or followed (Cond. 3), 50min apart, by a strong encoding event in the downstairs watermaze (red). Control conditions (2 and 4) replaced strong encoding events by weak encoding events (see arrows) in the downstairs watermaze. Memory for the different encoding events was assessed 24h (downstairs watermaze; red) or 26h (upstairs watermaze; black) after acquisition. ***B)*** Percentage of time spent searching the correct zone during probe trials. Stippled horizontal lines indicate chance level. Mean±1SEM. *Right*: Correlation of swim time percentage values obtained in both watermazes for the strong-before-weak condition (black) and the weak-before-strong (gray) conditions (Cond. 1 and 3). R^2 (R-squared).

Figure 3.13: Investigating a behavioural analogue of the "strong-before-weak" paradigm in the watermaze (Expt. 16)

A. Design (conditions)



B. Zone analysis (probe trials)

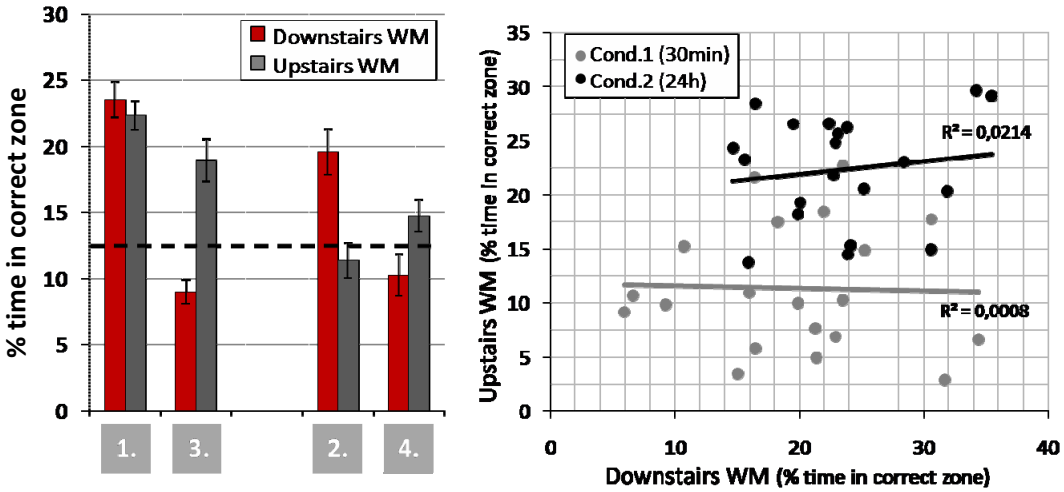
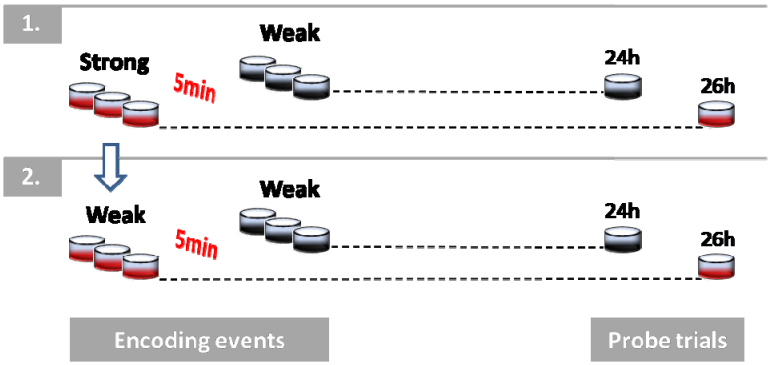


Figure 3.13. *Investigating a behavioural analogue of the "strong-before-weak" paradigm in the watermaze (Expt. 16).* ***A)*** Rats (n=20) were given a weak encoding event in the upstairs watermaze (black) preceded, 50min apart, by a strong (Cond. 1-2) or weak (Cond. 3-4) encoding event in the downstairs watermaze (red). Memory for the weak encoding events occurring upstairs was assessed 30min (Cond. 1 and 3) or 24h (Cond. 2 and 4) after acquisition. Memory for encoding events occurring downstairs (red) was assessed 26h after acquisition. ***B)*** Percentage of time spent searching the correct zone during probe trials. Stippled horizontal lines indicate chance level. Mean±1SEM. *Right*: Correlation of swim time percentage values obtained in both watermazes in conditions 1 and 2. R^2 (R-squared).

Figure 3.14: Investigating a behavioural analogue of the "strong-before-weak" paradigm in the watermaze (Expt. 17)

A. Design (conditions)



B. Zone analysis (probe trials)

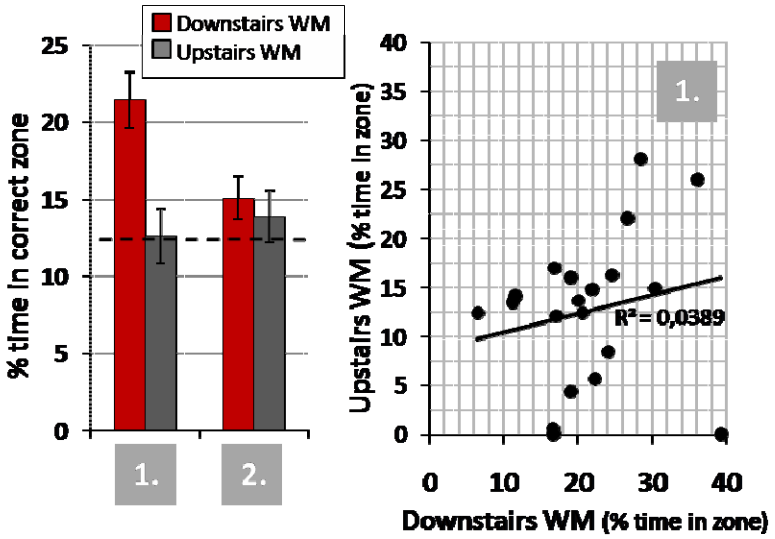


Figure 3.14. *Investigating a behavioural analogue of the "strong-before-weak"paradigm in the watermaze (Expt. 17).* ***A)*** Rats (n=20) were given a weak encoding event in the upstairs watermaze (black) preceded, 5min apart, by a strong (Cond. 1) or weak (Cond. 2) encoding event in the downstairs watermaze (red). Memory for the weak encoding events occurring in the upstairs watermaze was assessed 24h after acquisition. Memory for the encoding events occurring in the downstairs watermaze was assessed 26h after acquisition. ***B)*** Percentage of time spent searching the correct zone during probe trials. Stippled horizontal lines indicate chance level. Mean±1SEM. *Right*: Correlation of swim time percentage values obtained in both watermazes in condition 1. R^2 (R-squared).

Histological analysis revealed that the tips of the infusion cannulae were located in the dorsal hippocampi in all animals and that there was minimal damage to the tissue surrounding the cannulae and the injection sites (see Fig. 3.15e). Moreover, the animals performed normally during inter-probe days and during two training days given after the last probe day (see *Supplement 8a and 8b right*); this established that infusions of anisomycin did not produce permanent non-specific impairment of performance.

Inhibition of protein synthesis in the hippocampus following infusion of anisomycin (Expt. 19)

Autoradiographic imaging and quantitative densitometric analysis of [^{14}C] L-leucine uptake were used to assess the magnitude, diffusion and temporal decay of the protein synthesis inhibition resulting from the intra-hippocampal infusions of anisomycin used in behavioural experiments. Anisomycin and aCSF were infused simultaneously in opposed hippocampi to enable each rat to serve as its own control (see Fig. 3.16a); other than this, the infusion protocol was identical to that used in the behavioural experiments. [^{14}C] L-leucine was injected into the rats tail vein 30min (n=6), 3h45min (n=4), 6h45min (n=4), and 24h45min (n=4) after intra-hippocampal infusions, in order to reveal the extent of protein synthesis inhibition obtained at different time points that were critical for the behavioural experiments (e.g. start of encoding, retrieval, and training during subsequent days). Results showed that infusions of anisomycin produced a reversible inhibition of protein synthesis (detectable up to 6h45min but not 24h45min later) that was largely circumscribed to the dorsal pole of the hippocampus.

Quantitative analysis of autoradiographic images taken from coronal brain sections near the infusion site revealed that infusions of anisomycin virtually abolished the uptake of [^{14}C] L-leucine in dorsal hippocampal neurons after 30min. At this time point, [^{14}C] L-leucine uptake was reduced by 96-99% across hippocampal subfields (see Fig. 3.16b), with total hippocampal tissue tracer concentrations declining from a mean of 115±10nCi/g on the aCSF-injected side to

a mean of 2±2nCi/g on the anisomycin-injected side (see *Supplement 9*). Levels of protein synthesis inhibition in the hippocampus injected with anisomycin decreased to 81% after 3h45min, 45% after 6h45min, and 2% (not significant difference) after 24h45min. Inhibition of [^{14}C] L-leucine uptake was particularly persistent in the dorsal CA1-CA2 subfields. In these subfields, which are in close proximity to the infusion site, the levels of protein synthesis inhibition were still ~65-70% after 6h45min, nevertheless, "baseline" levels of tracer concentrations were observed in all hippocampal subfields after 24h45min. Analysis of more posterior brain sections (approximately 0.7mm from the infusion site), which included the ventral pole of the hippocampus, revealed that protein synthesis inhibition did not extend the full length of the dorso-ventral hippocampal axis. Considerable levels of inhibition were observed at the "posterior" sections of the dorsal hippocampus; however, the ventral hippocampus remained unaffected (see below). Overall, this contributed to the lower values of inhibition observed in posterior CA1-CA3 hippocampal subfields (see Fig. 3.16b, right). Quantification of tracer concentrations in the dorsal dentate gyrus (DG) also revealed that the magnitude and persistence of protein synthesis inhibition decreased along the anterior-posterior axis of the dorsal hippocampus. From anterior to posterior sections of the dentate gyrus the uptake inhibition values were reduced by 28% at 30min, 12% at 3h45min, and 29% at 6h45min (see Fig. 3.16b, right); no significant difference in tracer concentration values was observed between dentate gyri after 6h45min (see *Supplement 9*).

Figure 3.15: Dependence of long-term memory for "strong" encoding events on protein synthesis (Expt. 18)

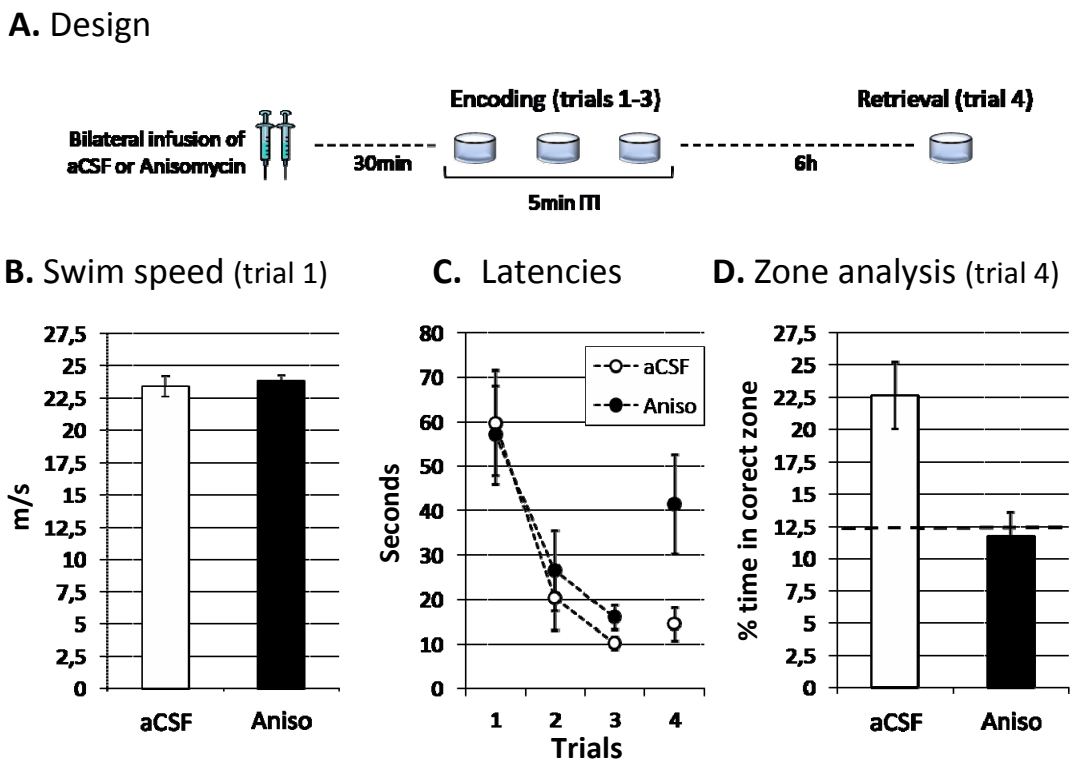
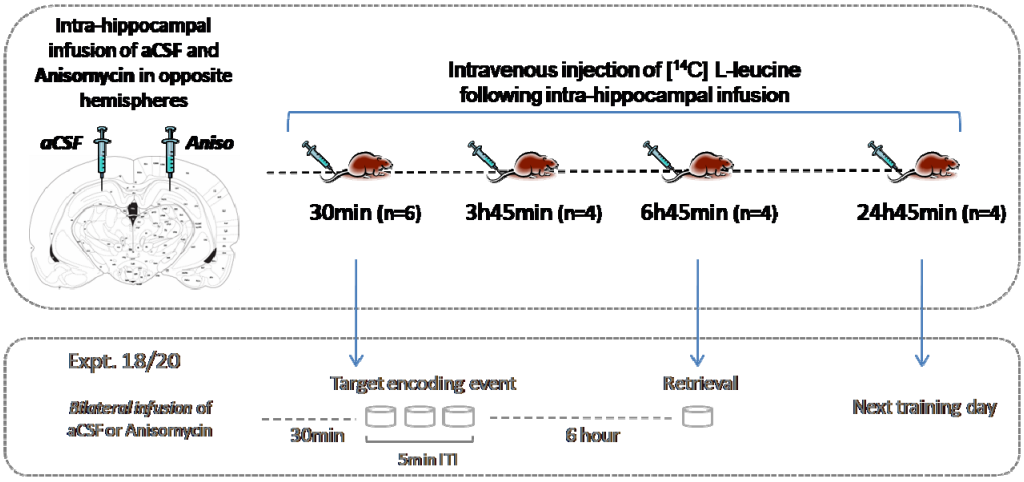


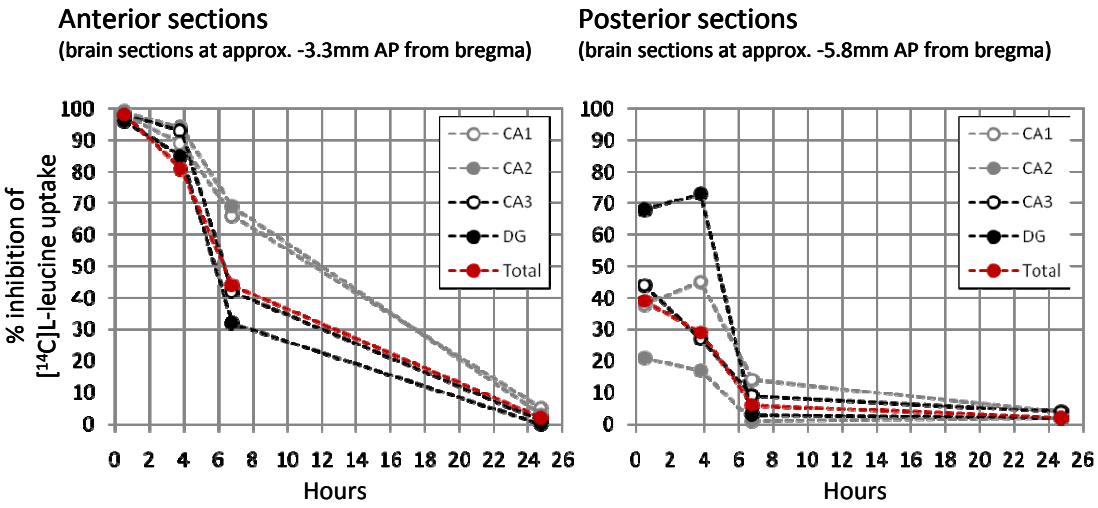
Figure 3.15. *Dependence of long-term memory for "strong" encoding events on protein synthesis in the hippocampus (Expt.18).* ***A)*** Rats (n=12) were given intra-hippocampal infusions of either vehicle (aCSF) or the protein synthesis inhibitor anisomycin (aniso; 125µg/µl; 1µl *per* side) 30min before the start of encoding. Encoding comprised 3 spaced swim trials (5min ITI). Memory for the "strong" encoding event was assessed after a retention interval of 6h. ***B)*** *Left*: Swim speed during the first encoding trial. *Centre*: Escape latencies obtained during the 3 encoding trials (Trial 1-3; dots connected by dashed lines; 5min ITI) and crossing latencies obtained during the probe trial 6h later (Trial 4). *Right:* Percentage of time that rats spent searching the correct zone during the probe trial (Trial 4). Stippled horizontal lines indicate chance level. Mean±1SEM. ***C)*** Photographs of two consecutive cresyl violet-stained coronal sections of a single animal (Rat nº8) showing correct placement of canullae in the dorsal hippocampus. These sections illustrate the absence of significant damage to hippocampal tissue, beyond that produced by cannulae placement, in an animal which showed good memory after aCSF infusions (29.8% time in correct zone) but no memory after administration of anisomycin; as well as no difference in T1 swim speed (26.2m/s with aCSF and 26.5 m/s with anisomycin).

Figure 3.16: Uptake of [^{14}C] L-leucine in the hippocampus following local infusion of aCSF or anisomycin (Expt. 19)

A. Design



B. Inhibition of [^{14}C] L-leucine uptake in the hippocampus following local infusion of the protein synthesis inhibitor anisomycin



C. Patterns of [^{14}C] L-leucine uptake in the hippocampus

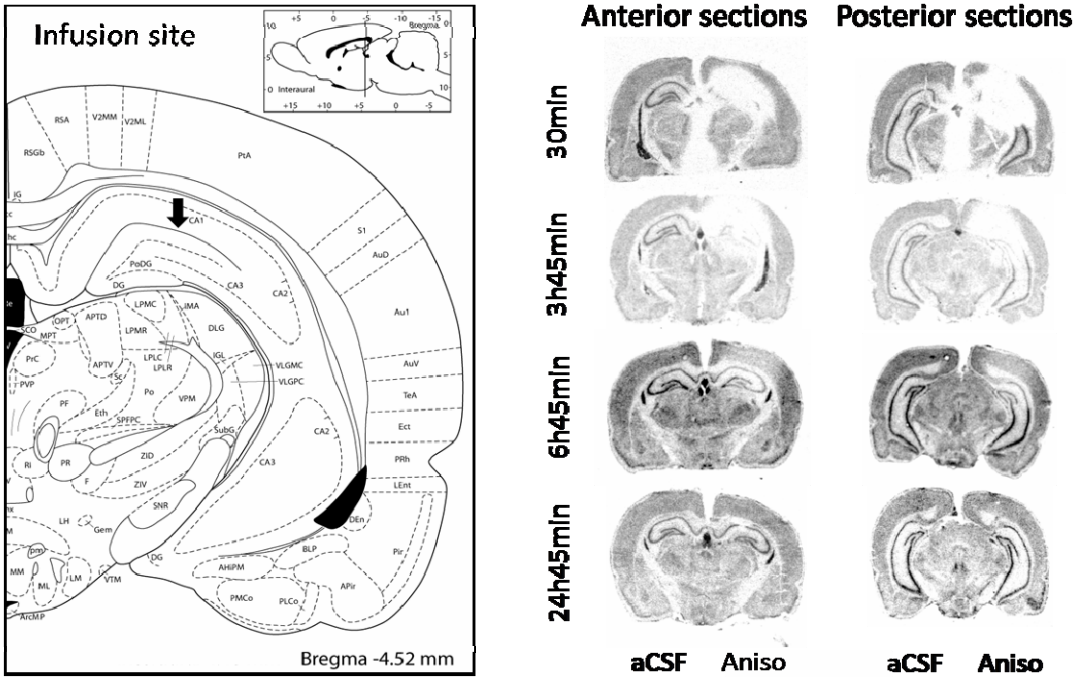


Figure 3.16. *Uptake of [^{14}C] L-leucine in the hippocampus following local infusion of aCSF or anisomycin (Expt. 19).* ***A)*** To establish the extent, spatial distribution and time course of protein synthesis inhibition produced by intra-hippocampal infusions of anisomycin, a bolus of [^{14}C] L-leucine was injected at a constant rate over 30s into the rats tail vein (7.5 µCi/100g) 30min (n=6), 3h45min (n=4), 6h45min (n=4), and 24h45min (n=4), after one hippocampus had been infused with anisomycin (125µg/1µl; 0.25µl/min) and the other, simultaneously, with aCSF. Time points were chosen to coincide with critical phases of the behavioural experiments (Expt. 18/20). ***B)*** Extent and time course of [^{14}C] L-leucine uptake inhibition in the hippocampus infused with anisomycin, as compared to the hippocampus infused with aCSF. To establish the extent of [^{14}C] L-leucine uptake inhibition along the anterior-posterior hippocampal axis, tracer concentrations were assessed in brain coronal sections localised at the level of the habenula (*left*; AP approx. -3.3mm) and the medial geniculate (*right*; AP approx. -5.2mm), to the infusion site. See Supplement 10 for absolute tracer concentration levels found in both hippocampi. ***C)*** *Left:* Diagram representing the infusion site (black arrow) in the dorsal hippocampus (coordinates from bregma: AP -4.5mm; ML 3mm; DV -3mm). Adapted from Paxinos and Watson (1998). *Right:* Representative brain coronal sections from 4 different animals injected with [^{14}C] L-leucine at the different time points. Sections were taken near the infusion site (*left*; anterior sections; AP approx. -4.5mm) and posterior to the infusion site (*right*; posterior sections; AP approx. -5.2mm).

Representative anterior and posterior coronal sections taken from animals sacrificed at the different time points are shown in Fig. 3.16c. In coronal sections near to the infusion site (*left*) inhibition of protein synthesis was mainly localized to the dorsal hippocampus. However, there was some evidence for inhibition in neocortical and thalamic regions. The same pattern of uptake distribution was observed in coronal sections posterior to the infusion site (*right*). Inhibition was restricted to the dorsal pole of the hippocampus, with tracer levels unaltered across ventral hippocampal subfields. In both anterior and posterior sections the higher density of the [^{14}C] L-leucine signal co-localized with hippocampal cell lines unaffected by anisomycin.

3.3.4. Investigating a behavioural analogue of the "strong-before-strong" paradigm in the watermaze (Expt. 20)

Experiment 18 established the dependence of long-term memory (6h) for "strong" encoding events (3 swim trials; 5min ITIs) on protein synthesis in the hippocampus. Conditions 1 and 2 of this experiment replicated those results but in the context of attempting another behavioural tagging experiment. Animals were given bilateral intra-hippocampal infusions of vehicle (aCSF) or the protein synthesis inhibitor anisomycin half an hour before strong encoding events (target events). Anisomycin, but not vehicle, disrupted the formation of long-term memory for those events. In conditions 3 and 4 the rats were given additional strong encoding events (modulatory events) preceding aCSF or anisomycin infusions by half an hour and target encoding events by one hour. Strong "modulatory" events producing memory detectable 8h later did not rescue the formation of long-term memory for strong "target" events occurring during protein synthesis inhibition.

Performance for strong "target" events in the upstairs watermaze (6h retention interval): As in Expt. 18, analysis of swim speed during encoding trials did not reveal a main effect of drug conditions (*data not shown*) rendering both zone analysis and analysis of crossing latencies viable measures of performance. Neither zone analysis ($F_{(1.14)}$<1) nor analysis of crossing latencies ($F_{(1.14)}$<1) revealed an interaction between number of encoding events (i.e. presence or no presence of "modulatory" events) and drug condition. Zone analysis revealed a clear effect of drug condition ($F_{(1.14)}$=11.0; p=0.005) on performance. Memory for "target" encoding events was only detected when rats were given infusions of aCSF (*Cond. 1 and 3:* $t_{(14)}$=2.4; p<0.05); infusions of anisomycin blocked the formation of long-term memory for these events independently of the presence of the "modulatory" events (*Cond. 2 and 4:* $t_{(14)}$<1) (Fig. 3.17b left, *black*). Although slightly higher crossing latencies were observed in conditions comprising inhibition of protein synthesis (*Cond. 2 and 4*) the difference failed to reach statistical significance ($F_{(1.14)}$<1; see *Supplement 10c black*). Importantly, neither zone analysis ($F_{(1.14)}$<1), nor analysis of crossing latencies ($F_{(1.14)}$<1), revealed a main effect of number of encoding events on performance. This suggests that memory for the "modulatory" events did not affect the formation of memory for the "target" events by mechanisms of proactive interference. The high levels of performance observed 8h after "modulatory" encoding events (Fig. 3.17b left, *red*) are puzzling when compared to those observed 6h after "target" events. This may reflect the use of different platform positions between watermazes, a "reminding" effect induced by retrieval of memory for the "target" events, and more unlikely, a general difference in performance between watermazes (see Expt. 21).

Performance for strong "modulatory" events in the downstairs watermaze (8h retention interval): Strong memory was observed for "modulatory" events in both conditions 3 and 4; accordingly, zone analysis revealed above chance levels of performance in both cases [($t_{(14)}$=4.9; p<0.001 and ($t_{(14)}$=6.7; p<0.0001), respectively; Fig. 3.17b left, red]. Importantly, neither zone analysis ($t_{(14)}$<1), nor analysis of crossing latencies ($t_{(14)}$<1; see *Supplement 10c red*), revealed a

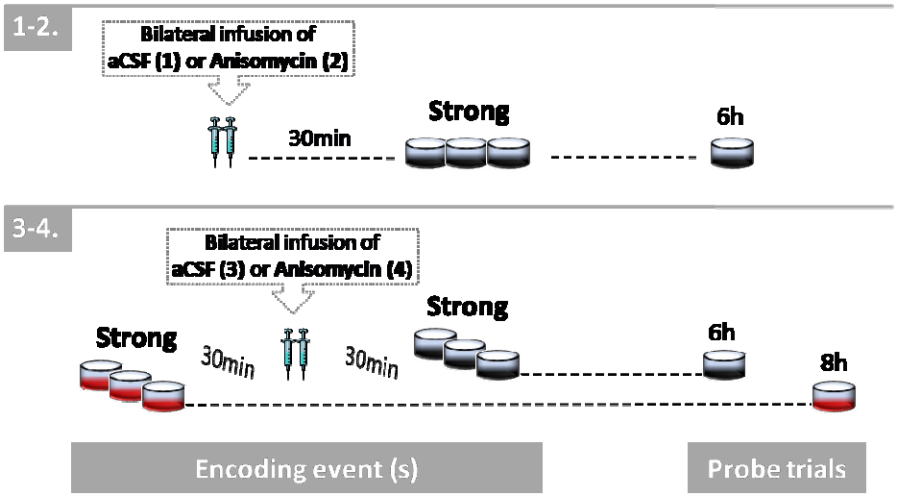
difference between conditions; suggesting that inhibition of protein synthesis, produced by the infusions of anisomycin, did not interfere with the formation of long-term memory for the preceding "modulatory" events.

Overall analysis: Neither zone analysis, nor crossing latencies, revealed a correlation between performance values obtained for "modulatory" and "target" encoding events in conditions 3 (*zone analysis:* $r_{(14)}$= 0.123, p=0.67; one-tailed; *crossing latencies:* $r_{(14)}$= 0.287; p=0.3056; one-tailed) and 4 (*zone analysis:* $r_{(14)}$= -0.305, p=0.27; one tailed; *crossing latencies:* $r_{(14)}$= 0.351; p=0.2046; one-tailed) (see Fig. 3.17b right and *Supplement 10c right*). Overall, these results do not support a behavioural correlate of the "strong-before-strong" synaptic tagging paradigm in the watermaze.

As in previous experiments, the histological analysis revealed that the tips of the infusion cannulae were located within the posterior dorsal hippocampi in all animals. Importantly, no significant damage was observed in the tissue surrounding the cannulae and the infusion sites. The fact that animals did not show impairments in performance during inter-probe days (see *Supplement 10*) further supports that consecutive infusions of anisomycin did not result in permanent non-specific impairment effects on learning and memory.

Figure 3.17: Investigating a behavioural analogue of the "strong-before-strong" paradigm in the watermaze (Expt. 20)

A. Design (conditions)



B. Zone analysis (probe trials)

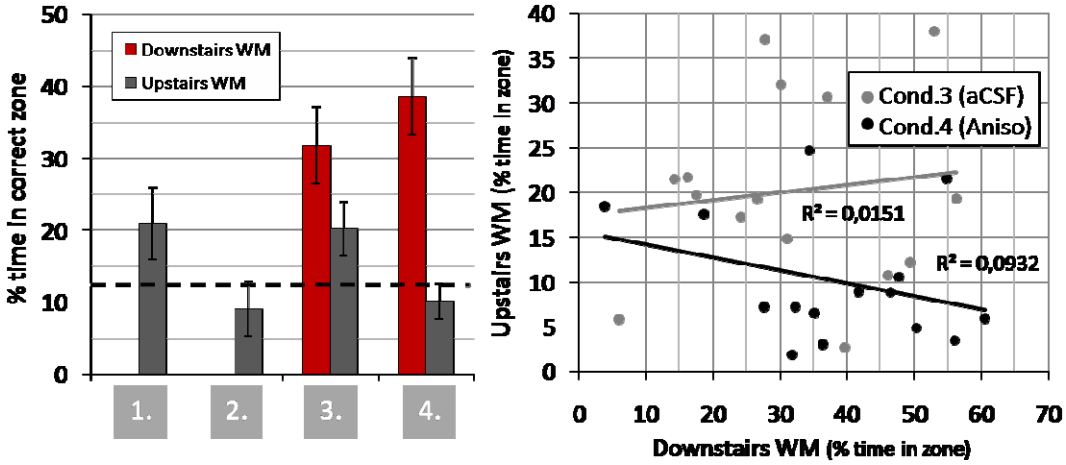


Figure 3.17. *Investigating a behavioural analogue of the "strong-before-strong" paradigm in the watermaze (Expt. 20).* ***A)*** Cond. 1-2: Rats (n=15) were given a strong encoding event in the upstairs watermaze (black) preceded (30min apart) by bilateral intra-hippocampal infusions of aCSF (Cond. 1) or anisomycin (Cond. 2; 125µg/µl; 1µl per side). Cond. 3-4: Rats were given two strong encoding events, one hour apart, in different watermazes located in different rooms. Bilateral intra-hippocampal infusions of aCSF (Cond. 3) or anisomycin (Cond. 4) occurred 30min after the first encoding event (downstairs watermaze; red) and before the second encoding event (upstairs watermaze; black). Memory for the strong encoding events occurring in the upstairs watermaze was assessed 6h after acquisition. Memory for the strong encoding events occurring in the downstairs watermaze was assessed 8h after acquisition. ***B)*** Percentage of time spent searching the correct zone during probe trials. Stippled horizontal line indicates chance level. Mean±1SEM. *Right*: Correlation of swim time percentage values obtained in both watermazes in conditions 3-4. R^2 – R-squared.

3.3.5. Investigating overlapping of neuronal ensembles recruited by two "strong" encoding events in the watermaze (Expt. 21)

Overlapping of neuronal ensembles recruited by two "strong" encoding events could not be determined because no significant number of cells containing *Arc* intra-nuclear foci, which would reflect neuronal activation during Epoch 2 (and 2nd encoding events), was obtained in any of the experimental groups [see Fig. 3.18b (*centre*) and 3.18c (left)]. Previous studies have shown that a mean percentage of total Arc^+ cells (i.e. including $H1a^+/Arc^+$ cells) lower than 5% corresponds to baseline levels of cell activation in the CA1 hippocampal region obtained in caged control animals (e.g. Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004). In this experiment, the mean percentage of total Arc^+ cells obtained in all experimental groups was never superior to 3%. Expression of cells containing *H1a* INF was significantly up-regulated in animals trained in the watermaze during Epoch 1 [see Fig. 3.18b (*left*) and 3.18c (*left*, Groups 3 and 5)]. A main effect of experimental group on total $H1a^+$ cell counts (also including $H1a^+/Arc^+$ cells) was revealed by means of ANOVA ($F_{(4,15)}$=13.45; $p\leq0.0001$) and *post-hoc* comparisons revealed significant differences between caged controls (Cond. 1) and trained animals (Cond. 3/5; $p\leq0.0005$), but no difference between caged controls and "moved" controls (Cond. 2/4; p=0.37). Total $H1a^+$ cell counts for animals trained in a single watermaze during Epochs 1 and 2 were significantly higher than those obtained for animals that were simply moved to a single holding room during those two time periods ($p\leq0.005$). Likewise, total $H1a^+$ cell counts for animals trained in two different watermazes were significantly higher than those obtained in animals moved to different holding rooms ($p\leq0.0005$). No difference was observed between animals trained in one, or two, watermazes during Epochs 1 and 2 (p=0.18). Overall, these results suggest that induction of *H1a* mRNA in CA1 hippocampal neurons was training-specific and not due to any other factor intrinsic to testing procedures. Despite the low counts of total Arc^+ cells, which prevent the proper quantitative estimation of overlap between activated neuronal ensembles, there were some cells expressing both *H1a* and *Arc* intra-nuclear foci

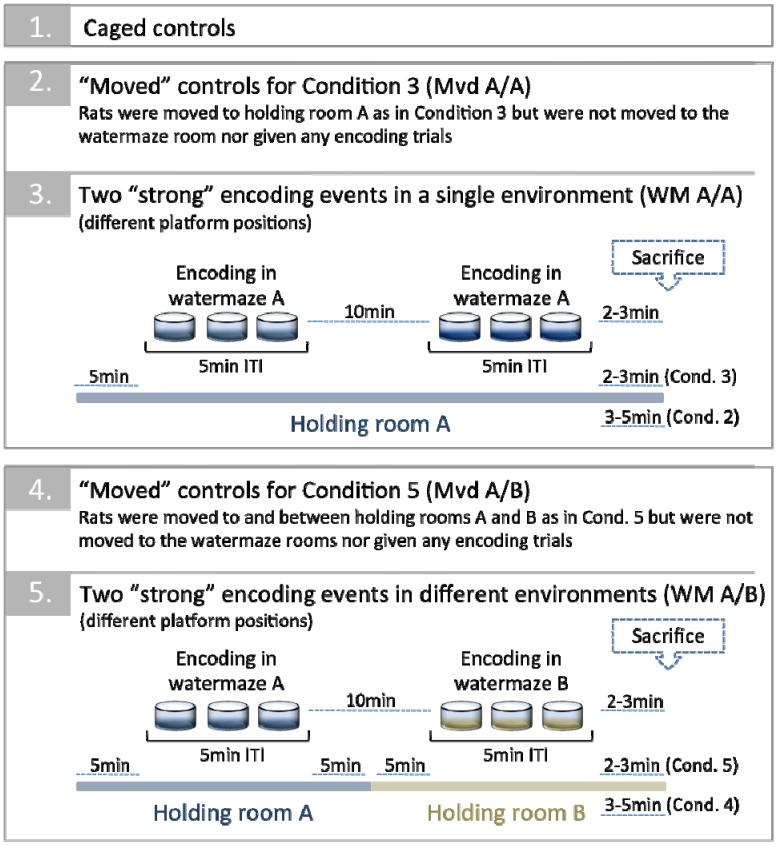
($H1a^+/Arc^+$ cells). Counting of these cells provided some indication that, as it would be expected, the level of overlap obtained between cells activated by training in two different environments (Cond. 5) was lower than that obtained between cells activated by repeated training in a single environment (Cond. 3; $F_{(1,6)}$=6.58; $p \leq 0.05$).

3.3.6. Up-regulation of Arc protein in CA1 hippocampal neurons following strong encoding events in the watermaze (Expt. 21)

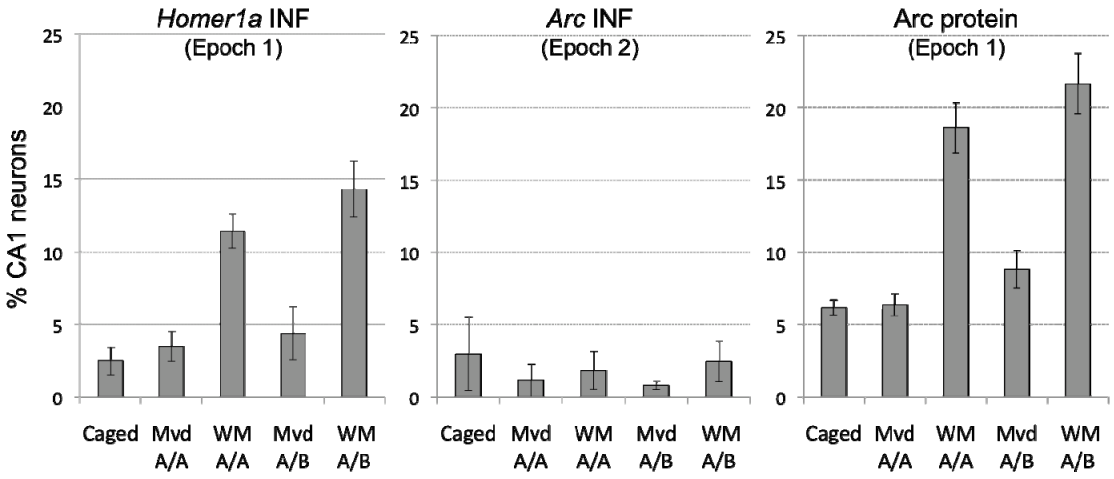
Expression of Arc protein, reflecting neuronal activation 30-90min before sacrifice (which overlaps with Epoch 1), mirrored the pattern of expression of *H1a* mRNA (see Fig. 3.18b and c; *right*). As observed with *H1a* mRNA, significant up-regulation of Arc protein was only obtained in animals given strong encoding events during Epoch 1. An overall ANOVA revealed a main effect of group on Arc^+ cell counts ($F_{(4,15)}$=26.37; $p \leq 0.001$). *Post-hoc* comparisons revealed significant differences between caged controls (Cond. 1) and animals trained in the watermaze (Cond. 3/5; $p \leq 0.0001$). No differences were found between caged and "moved" controls (Cond. 2/4; p=0.21). Arc^+ cell counts for animals trained in a single watermaze were significantly higher than those observed in animals moved to a single holding room ($p \leq 0.001$). Arc^+ cell counts for animals trained in two different watermazes were also significantly higher than those obtained for their respective "moved" controls ($p \leq 0.0001$). No significant difference was observed between groups of animals given encoding events in one or two watermazes during Epochs 1 and 2 (Cond. 3 and 5; p=0.15). Overall, these results suggest that strong encoding events in the watermaze during Epoch 1 led to the coordinated induction of both *Arc* and *H1a*, and significant expression of Arc protein, in CA1 hippocampal neurons.

Figure 3.18: Investigating overlapping of CA1 neuronal ensembles recruited by "strong" encoding events in the watermaze (Expt. 21)

A. Design (conditions)

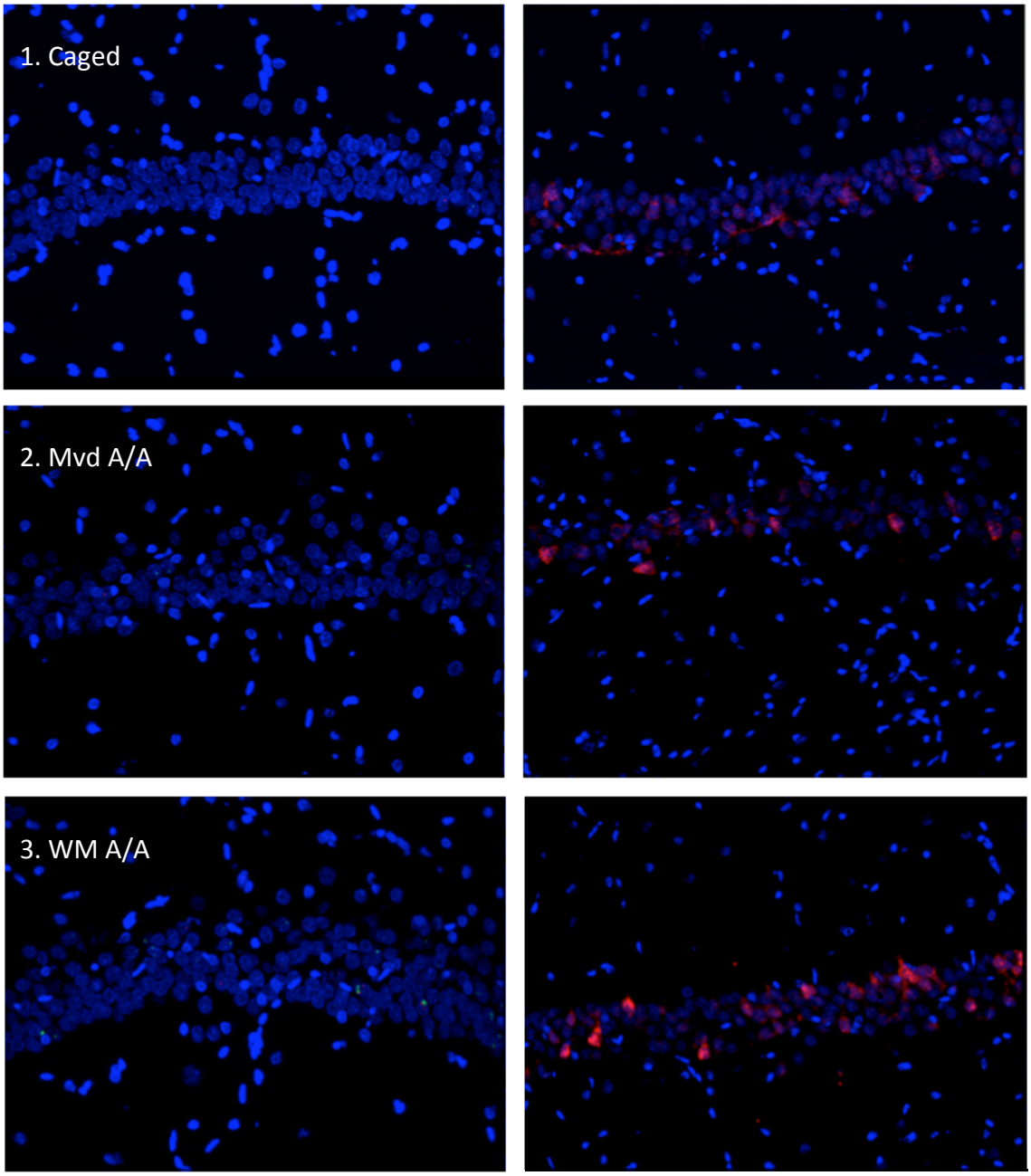


B. Up-regulation of *Homer1a* mRNA and Arc protein in CA1 hippocampal neurons following strong encoding events in the watermaze



C. Confocal microscope images of CA1 neurons expressing intranuclear *H1a* and *Arc* foci and cytoplasmic Arc protein

H1a/*Arc* INF | Arc protein



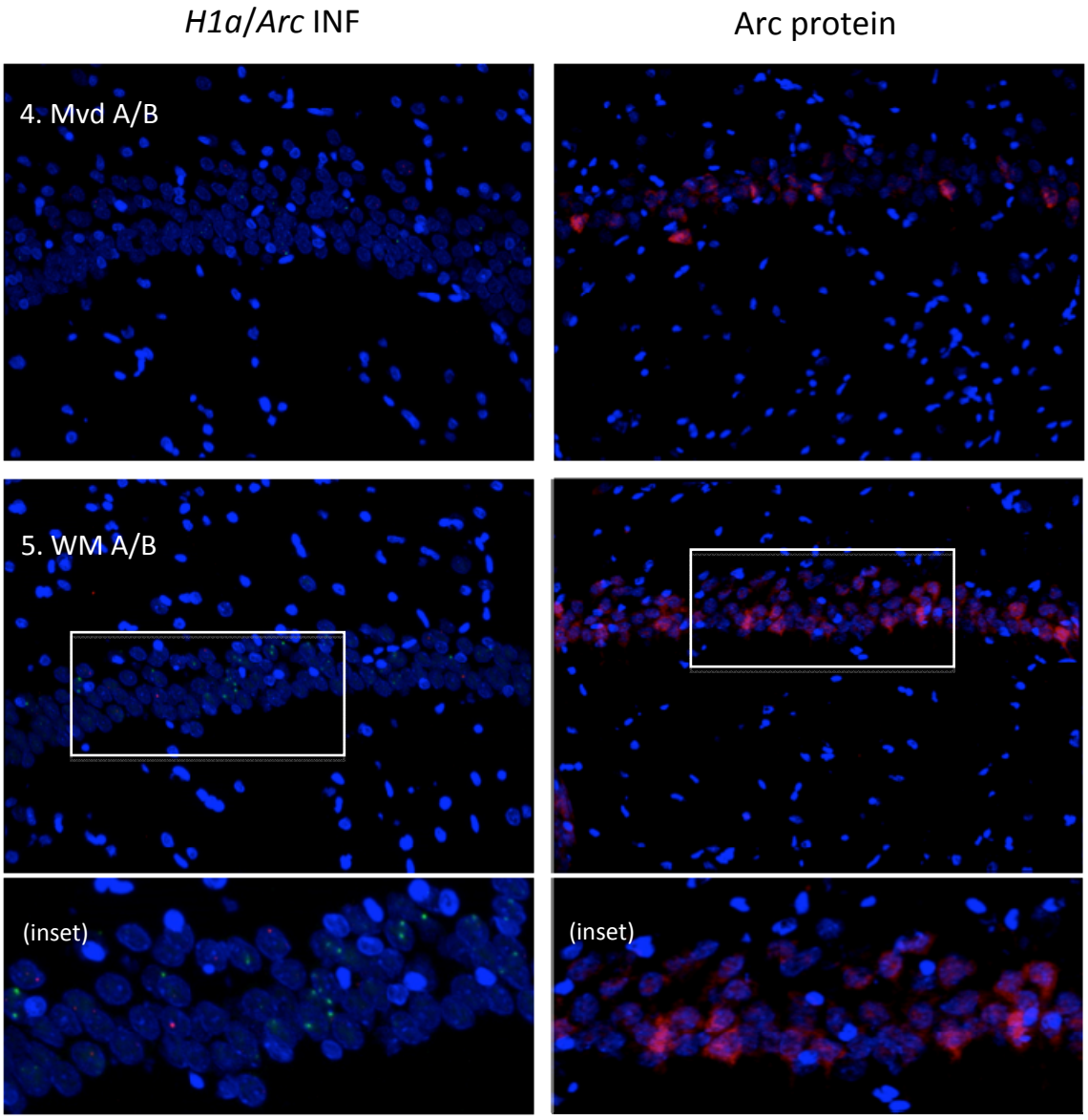


Figure 3.18. *Investigating the overlap of neuronal populations recruited by two "strong" encoding events occurring in two different environments (Expt. 21).* ***A)*** *Design.* Cond. 1 (*caged controls*): Rats were sacrificed directly from their home cages. Cond. 3 and 5 (trained groups): Rats were given two "strong" encoding events in a single watermaze (Cond.3) or each in a different watermaze (Cond.5). The animals were moved to the watermaze holding rooms 5min before the first encoding trial. In Cond. 5 the time spent in the holding rooms was matched between different watermazes. Cond. 2 and 4 (*moved controls*): Rats were moved between holding rooms as in Cond. 3 and 5 but not given encoding trials. ***B)*** Percentage of CA1 cells containing *H1a* or *Arc* intranuclear foci (including $H1a^{+}/Arc^{+}$ cells) and Arc protein. Mean±1S.E.M. (*n*=4 per group). *Left:* Significant up-regulation of nuclear H1a mRNA was only observed in animals given strong encoding events in the watermaze during Epoch 1 [Cond. 3 (WM A/A) and 5 (WM A/B)]. *Center:* No significant expression of *Arc*+ cells (which refers to Epoch 2) was observed in any of the experimental groups. This prevented the analysis of overlap between neuronal ensembles recruited by strong encoding events during Epochs 1 and 2. *Right:* The pattern of Arc protein expression was similar to that obtained for *H1a* intranuclear foci; only animals given strong encoding events in the watermaze during Epoch 1 revealed significant up-regulation of Arc protein. ***C)*** Representative confocal microscope images showing expression of *H1a* (green) and *Arc* (red) intranuclear foci (INF; *left*) and cytoplasmic Arc protein (red; *right*), in CA1 hippocampal neurons of animals allocated to the different experimental groups. Nuclei are stained in blue. Each image is the collapsed image stack through 20µm of tissue and corresponds to a scale of 447x335µm; insets are an amplification of some of the cells of adjacent images and are therefore in a different scale.

Chapter 4: Discussion

4.1. Overview

The experimental research presented in this dissertation represents a first effort to investigate the contribution of synaptic tagging and capture mechanisms to the formation of long-lasting allocentric place memory.

First, two new behavioural tasks were developed in order to *i)* provide sensitive measures of allocentric place memory strength and persistence and to *ii)* characterize behavioural and neural processes underlying the acquisition and stabilisation of place memory driven by appetitive or aversive motivation. One-trial place memory was shown to decline rapidly and monotonically, within minutes to hours of acquisition, in both tasks. In the new food rewarded "event arena" task, encoding but not retrieval of memory acquired in a single encoding trial depended on the activation of NMDA receptors in the hippocampus. Activation of hippocampal AMPA receptors was required for memory retrieval. It was also confirmed that, infused into the hippocampus exactly as in the behavioural studies, the NMDA receptor antagonist D-AP5 selectively blocked LTP induction, whereas the AMPA receptor antagonist CNQX reduced fast excitatory transmission at perforant-path dentate gyrus synapses. In the modified delayed

matching-to-place (DMP) task in the watermaze, the strength and persistence of aversively motivated place memory were further shown to depend on the nature, number, and temporal distribution of acquisition trials. Placement trials, in which animals were only placed on the escape platform for 30s, produced weaker memory than standard trials in which animals had to swim to escape to the platform. Finally, repetition and/or spacing of standard acquisition trials were shown to improve the strength of long-term place memory.

Second, new training protocols were developed using the watermaze DMP task to investigate if STC-like mechanisms could be detected during the formation of long-lasting allocentric place memory. *Strong* encoding events, up-regulating both transcription and translation in the dorsal hippocampus and producing long-lasting memory requiring protein synthesis in that same brain region, were first combined with *weak* encoding events (only producing short-lasting memory), similarly to "*strong-before-weak*" and "*weak-before-strong*" paradigms characterized in electrophysiological studies *in vitro*. Contrary to the prediction of the STC hypothesis, memory for *weak* encoding events did not become long-lasting when these were preceded, or followed, one hour apart, by *strong* encoding events occurring in a different environment. A behavioural correlate of the "*strong-before-strong*" paradigm was also not observed, as memory for *strong* encoding events occurring during translational arrest in the hippocampus was not rescued by different *strong* encoding events occurring in a different environment one hour earlier.

Overall, these results presented in this thesis are supportive of ***i)*** a role for hippocampal NMDA receptor-mediated synaptic plasticity in the encoding of rapidly acquired allocentric place memory; ***ii)*** a role for hippocampal AMPA receptor-mediated synaptic transmission in both encoding and retrieval of memory; ***iii)*** and a role for transcriptional and translational mechanisms in the hippocampus in the stabilisation of memory. However, ***iv)*** no evidence was secured for the involvement of STC-like mechanisms in the formation of long-lasting allocentric place memory.

4.2. The new one-trial place memory task in the event arena

In the one-trial place memory task in the event arena rats must encode a memory of a trial-specific place in a familiar environment and, after a retention interval, retrieve this memory to efficiently obtain food reward in an open arena. Performance in probe trials, with food omitted from the sandwells during retrieval, demonstrated no reliance on food odours. Rats may use both relationships among multiple environmental cues and elemental cues, such as odour traces and directional or idiothetic cues, to find a place (Eichenbaum et al., 1990; Hodges, 1996; Jacobs and Schenk, 2003a). The different start positions for encoding and retrieval phases rendered idiothetic or directional cues unhelpful in this task. The disruption of retrieval in the dark and the lack of an effect of arena rotation further established the importance of visual cues. Altogether, it is warranted to conclude that the task requires visuo-spatial relational memory based on a single experience. The protocol resembles tests of viewpoint-independent place memory that were developed to study aspects of episodic memory and hippocampal function in humans (Holdstock et al., 2000; Burgess et al., 2002). It complements one-trial place memory tasks in the water maze (Morris, 1983, 1984; Panakhova et al., 1984; Whishaw, 1985; Steele and Morris, 1999), which require aversively motivated escape and have very different sensorimotor demands, and in the radial-arm maze (Olton and Samuelson, 1976; Alexinsky and Chapouthier, 1978; Sinnamon et al., 1978; Floresco et al., 1997; Lee and Kesner, 2002), which favour the use of directional and idiothetic cues (Hodges, 1996; Dubreuil et al., 2003; Jacobs and Schenk, 2003b). The task also represents an extension of procedures developed in the cheeseboard apparatus (Gilbert et al., 1998; Gilbert et al., 2001; Kirwan et al., 2005). These rely on correct first choices accumulated over many trials to measure one-trial place memory, whereas in the event arena paradigm, the dig-time and error measures allow the assessment of performance on discrete trials, a prerequisite for the separate study of encoding and retrieval mechanisms.

4.3. Contribution of hippocampal glutamate receptor activation for encoding and retrieval of one-trial allocentric place memory

In the event arena task, D-AP5 infusion before encoding resulted in chance performance, whereas infusion after encoding left performance intact. These results extend findings of other studies, using pharmacological, molecular, and genetic techniques, that hippocampal NMDA receptor activation is required for one-trial place memory (Steele and Morris, 1999; Lee and Kesner, 2002; Nakazawa et al., 2003). Specifically, they reveal that these receptors contribute to the encoding but not retrieval of such memory. These findings do not speak to the claimed role of hippocampal NMDA receptors in memory consolidation after encoding (Packard and Teather, 1997; Shimizu et al., 2000; Rossato et al., 2004), because there was no sufficiently delayed retrieval test. There is also a debate about such a role for NMDA receptors (Day and Langston, 2006).

In vitro experiments have established that hippocampal NMDA receptors are critical for the induction of LTP but of minor importance for basal synaptic transmission in the hippocampus (Bliss and Collingridge, 1993). *In vivo* recordings demonstrated that hippocampal D-AP5 infusion impairs the induction of hippocampal LTP (Errington et al., 1987; Morris et al., 1989; Lee and Kesner, 2002), but marked reductions in basal synaptic transmission have also been reported (Walker and Gold, 1994). Recordings from perforant-path dentate gyrus synapses in urethane-anesthetized rats established that D-AP5, infused into the hippocampus exactly as in our behavioural experiments, selectively blocked LTP induction. Comparable effects likely occurred at excitatory synapses in subfields CA1–CA3, because these synapses (except for mossy fibre synapses) show NMDA receptor-dependent LTP (Bliss and Collingridge, 1993; Lee and Kesner, 2002) and CA1–CA3 and dentate gyri were similarly close to the infusion sites; moreover D-AP5, infused as in the present study, diffuses evenly throughout the dorsal hippocampal subfields (Steele and Morris, 1999). Altogether, these results are consistent with

the notion that NMDA receptor-mediated induction of hippocampal synaptic plasticity at several sites contributes to the encoding of one-trial place memory. Interestingly, induction of LTP at intrinsic hippocampal pathways creates new hippocampal place fields that are stable for about 6h (Dragoi et al., 2003); such place fields may be neural correlates of the place representations underlying performance in the task. CNQX infusion before retrieval impaired performance, and CNQX reduced fast excitatory transmission at perforant-path dentate gyrus synapses; comparable effects probably occurred at other intrinsic hippocampal synapses [as demonstrated for the AMPA receptor antagonist LY326325 (Riedel et al., 1999)]. Retrieval was not blocked completely. This may reflect that, even close to the infusion site, excitatory synaptic transmission was reduced by only ~50% at the time of testing (Fig. 2.7a). Although CNQX infusions before encoding were not investigated, AMPA receptor-mediated synaptic transmission is necessary to enable NMDA receptor-mediated hippocampal encoding mechanisms (Bliss and Collingridge, 1993). These findings are consistent with the idea that hippocampal AMPA receptors, being critical for basal synaptic transmission (Davies and Collingridge, 1989; Lambert and Jones, 1990), contribute to both encoding and retrieval of place memory (Riedel et al., 1999).

The present findings are consistent with notions holding that rapid encoding of a stimulus pattern into relational memory, such as allocentric place memory, requires the induction of hippocampal synaptic plasticity, and the subsequent retrieval requires excitatory hippocampal transmission to activate the stored pattern representation after perceiving a part of this pattern. Additionally, hippocampal synaptic transmission may mediate the use of hippocampal memory by relating it to mechanisms of response and motor control via hippocampal connections to sub-cortical sites and the prefrontal cortex (Floresco et al., 1997; Bast and Feldon, 2003; Bannerman et al., 2004; Peleg-Raibstein et al., 2005; Bast, 2007). In contrast, rats can incrementally acquire place memory and later retrieve it despite impaired hippocampal synaptic plasticity (Bannerman et al., 1995; Saucier and Cain, 1995; Reisel et al., 2002; Schmitt et al., 2003) and even with the

hippocampus completely lesioned (Morris et al., 1990; Hunt et al., 1994). Alternative accounts are less consistent with the present findings. The particular sensitivity of one-trial place learning to treatments blocking hippocampal LTP argues against a function of hippocampal LTP-like synaptic plasticity as an "attentional device" (Shors and Matzel, 1997), because this would imply that blockade of synaptic plasticity should not impair one-trial learning [as outlined by (Fanselow, 1997)]. State dependency, the dependence of memory retrieval on the congruity between brain states during encoding and retrieval (Overton, 1964; Izquierdo, 1984), is also unlikely to account for the results. AP-5 infusions before encoding prevented retrieval 20min later when the drug was still active in the hippocampus (as evidenced by blockade of LTP induction 2–3h after infusion; unpublished observations). D-AP5 infusions before retrieval left performance intact, despite encoding in a drug-free state. Finally, the different effects of AP-5 infusions before and after encoding and the absence of consistent gross sensorimotor impairments or differences in overall dig time, both after D-AP5 and after CNQX infusions, argue against the possibility that the infusions interfered with motivational and sensorimotor task demands.

In radial-arm maze experiments, rats acquired and maintained trial-specific place information in a familiar environment over retention intervals of minutes to hours independent of hippocampal NMDA receptors (Shapiro and O'Connor, 1992; Caramanos and Shapiro, 1994; Kesner and Rolls, 2001). This may reflect that representations of elemental stimuli, such as idiothetic and directional cues, supported place memory. Furthermore, in the radial arm maze, there are typically only eight places potentially containing reward (compared with 32 in the event arena), so that the rats become highly familiar with these places throughout training. Under these circumstances, attractor states of hippocampal activity representing the familiar places may be established during training and support one-trial place memory with no further induction of synaptic plasticity necessary during later trials (Kesner and Rolls, 2001).

4.4. Persistence of one-trial allocentric place memory

One-trial place memory strength declined monotonically with increasing retention intervals in both the watermaze and event arena DMP tasks. This was particularly evident with the zone analysis and the dig-time measures of search preference, which revealed comparable rates of memory decay (see Fig. 4.1a). These results are not supportive of the view that the nature of motivation of allocentric place memory determines different rates of decay over time (Bolhuis et al., 1985). In both tasks memory was shown to decay rapidly within minutes to hours of acquisition, with weak, but still detectable, memory observed after 6h.

One striking finding in these studies was the different sensitivity of the several measures of performance used in these two tasks to variations in memory strength and persistence. While measures of search preference revealed rapid forgetting, other measures such as escape latencies in the watermaze, and first choices and errors in the event arena, were more resistant to the effect of increasing retention intervals. Differences in the intrinsic variability of these behavioural measures may justify their different sensitivity to variations in memory strength. As an example, Fig. 4.1b shows the relative variability of the different performance measures used in Expts. 8-10 to assess persistence of one-trial place memory in the watermaze. For all retention intervals, the measure of performance showing the lower variability was the zone analysis, followed by retention trial (T2) latencies and, finally, escape latency savings. In view of this, the sensitivity of these measures to variations in memory strength declined as a function of their variability, i.e. decay of memory was clearly revealed by zone analysis, less by T2 latencies and not detected by escape latency savings. The higher variability in T2 latencies and escape latency savings may have been introduced by chance factors, e.g. when the rat “bumps” into the platform unexpectedly, with additional variability of savings most likely resulting from the fact that it also depends on performance during encoding trials, when animals are searching for an unknown platform location. Another issue concerning escape latencies and path lengths

that may justify their resistance to the effect of increasing retention intervals is the fact that they may be efficiently reduced through systematic search strategies and the use of single beacon cues (e.g. Morris, 1981; Buresova et al., 1985a; Schenk and Morris, 1985; Jacobs and Schenk, 2003b). Also, in the event arena task, the higher variability of first choices and errors may justify their lower sensitivity to variations in memory strength (see Fig. 4.1c). Chance factors, such as when the animal runs into the sandwells that are closer to the start box, may again contribute to such variability. Overall, these results suggest that the use of different behavioural measures may explain the different accounts of one-trial place memory persistence in the watermaze (Panakhova et al., 1984; Morris et al., 1990; Steele and Morris, 1999; von Linstow Roloff et al., 2002; de Hoz et al., 2005; O'Carroll et al., 2006). In these studies, as in the present experiments, retention trial latencies proved to be more sensitive than either latency or path length savings to the effect of increasing retention intervals. Comparable rates of memory decay were also observed between those studies and the present study when assessed by latency-based measures of performance. Importantly, the present experiments reveal that the introduction of search preference as a measure of performance in the watermaze DMP task further improves its sensitivity to variations in memory strength, namely, to forgetting over time.

That different measures of performance may reflect different rates of memory decay in the watermaze was also evidenced by a recent study by Bolding and Rudy (2006). In this study the authors reported that memory for 10 consecutive trials (2min ITI) could be detectable up to 4 hours when using the conventional quadrant measure of search preference, but only up to 30min when a *difference score index of selective search* (which compares the time that rats spend in the target quadrant with the time that they spend in their second most preferred quadrant) was used. In the present study a single acquisition trial was shown to produce selective search detectable up to at least 6h. Procedural differences are the most likely explanation for divergences between studies. In the present study rats were pretrained in the task previous to testing, rats in the Bolding and Rudy study were only given 10 trials within a day. Pretraining is

a critical period in which animals are given the opportunity to master the procedural (non-spatial) requirements of the task. Task acquisition itself is known to induce a strong stress response that is gradually reduced over training days. For example, rats given 10 trials a day for 1, 3 or 5 days, and sacrificed 5-60min after the last trial, have been shown to have a strong hormonal stress response (including increased serum costicosterone levels) by day 1 that normalized within the following days (Aguilar-Valles et al., 2005). Strong stress and high levels of corticosterone impair the retention of memory in the watermaze (e.g. de Quervain et al., 1998) and may have been one of the factors contributing to the poor memory observed after 10 training trials.

4.5. Induction of transcriptional and translational mechanisms in the hippocampus following spaced training in the watermaze

As referred to before, multi-trial spaced training has been widely established to facilitate long-term retention of memory in rodents (e.g. Glickman, 1961; Deutsch, 1962; McGaugh, 1966; Goodrick, 1973; Mitchell, 1973; Domjan, 1980; Roberts and Dale, 1981; Fanselow and Tighe, 1988; Kogan et al., 1997; Josselyn et al., 2001; Genoux et al., 2002; Scharf et al., 2002), namely in the watermaze (Morris and Doyle, 1985; Kogan et al., 1997; Spreng et al., 2002; Bolding and Rudy, 2006; Sisti et al., 2007). The mechanisms underlying the "spacing effect" are still unclear (Eichenbaum, 1997b) but research on both synaptic plasticity and memory consolidation suggest that they engage and potentiate transcriptional and translational machinery (Kogan et al., 1997; Josselyn et al., 2001; Genoux et al., 2002; Scharf et al., 2002). In this study, spaced multi-trial training was shown to facilitate the formation of long-term memory in the watermaze (see Expt. 11) and "strong" encoding events comprising spaced encoding trials were shown to induce both transcriptional and translational mechanisms in the dorsal hippocampus (see Expt. 21). Up-regulation of protein synthesis was a requirement for strong encoding events designed to test behavioural correlates of STC mechanisms.

Figure. 4.1: Comparison of one-trial place memory persistence in the "event" arena and the watermaze DMP tasks

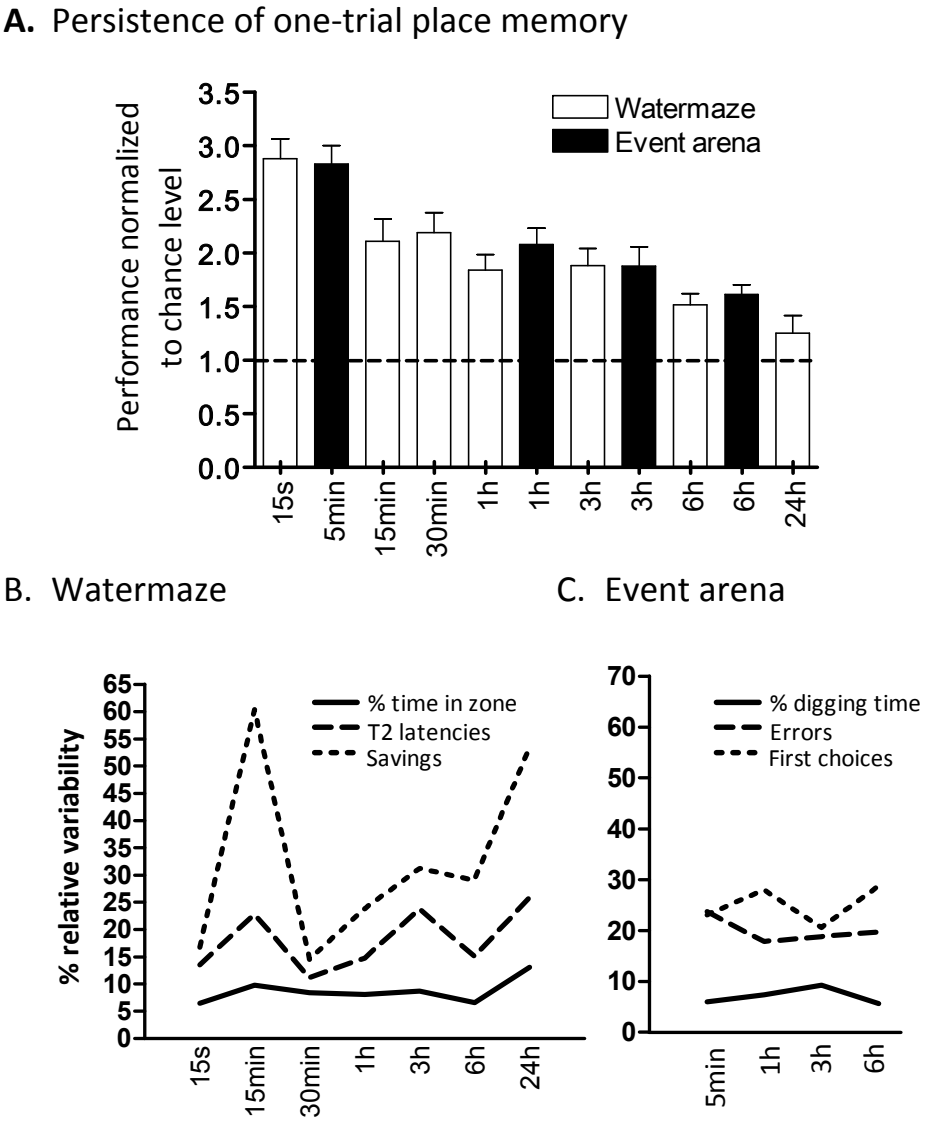


Figure 4.1. Comparison of one-trial place memory persistence in the "event" arena and the watermaze tasks and relative variability of performance measures. ***A)*** Normalization of the watermaze data to chance was calculated as the percentage of time swimming in the correct zone divided by the chance level (12.5%). Normalization of the event arena data was calculated as the percentage of time digging in the correct sandwell divided by the chance level (20%). Watermaze data points at 15s, 1h and 6h comprise the average of performance levels obtained in experiments 8-10. Stippled horizontal line indicates chance value of normalized performance measures. Mean±1SEM. ***B)*** The percent relative variability of the different measures of performance was calculated as the standard error of the mean divided by the absolute value of the mean and multiplied by 100. Watermaze data points at 15s, 1h and 6h comprise the average of the performance levels obtained in experiments 8-10.

Strong encoding events in the watermaze were shown to induce expression of Homer 1a mRNA and Arc protein in CA1 hippocampal neurons. Induction of both Homer 1a mRNA and Arc protein in the hippocampus has been implicated in the consolidation of both hippocampal synaptic plasticity and hippocampal-dependent memory (see Introduction). In the context of this study it is important to stress that transport and input-specific delivery of Homer 1a mRNA to hippocampal synapses have also been quite recently shown to assemble all the characteristics of a STC mechanism (Okada et al., 2009). Importantly, training and formation of long-term memory in the watermaze have been associated not only to the expression of Arc protein (Guzowski et al., 2000), but also to the expression of Arc mRNA (Guzowski et al., 2001; Gusev et al., 2005) in the hippocampus. In this study, training in the watermaze did not produce detectable expression of Arc mRNA in CA1 hippocampal neurons (see Expt. 21). The low percentage of Arc^{+} cells in the present experiments is not attributable to technical issues: *First,* the fact that $H1a^{+}$ cells were clearly detected in the tissue makes RNA degradation during tissue collection, shipment, or processing, an unlikely explanation. This was further supported by the fact that the few detected *Arc* INF were clear and bright [see 3.18c; *left*, inset for Group 5)], which would not be observed if RNA was degraded. *Second*, the low percentage of total Arc^{+} cells was consistently obtained with different types of riboprobe labelling (*Arc*-Flu, *Arc*-DNP and *Arc*-Dig), excluding riboprobe sensitivity as a viable explanation. A possible explanation is that strong encoding events did induce expression of Arc mRNA, but only during the earlier part of training (Miyashita et al., 2009). As Arc^{+} INFs are expressed approximately 2-15min after induction and the strong encoding events used in this study had the approximate duration of 15min, it is possible that the Arc^{+} INFs induced only by initial training trials were gone by the time animals were sacrificed. This possibility was not anticipated in the design of the experiment and has been corrected in ongoing experiments using catFISH to reanalyse the overlap of neuronal ensembles recruited by two distinct encoding events.

4.6. Dependence of long-term allocentric place memory on translational mechanisms in the hippocampus

Bilateral intra-hippocampal infusions of the broad specific protein synthesis inhibitor anisomycin were used to investigate the requirement for translational mechanisms in the hippocampus during the formation of long-term place memory in the watermaze. The central administration of this drug prevented the likelihood of unwanted effects on physiology and behaviour associated to systemic administration and enabled the identification of a main target of action. As referred in the introduction, the effect of both systemic and central administration of anisomycin on protein synthesis has been well characterised across species and brain structures (Flood et al., 1973; Davis et al., 1980; Patterson et al., 1989; Rosenblum et al., 1993; Meiri and Rosenblum, 1998; Maren et al., 2003; Ben Mamou et al., 2006; Morris et al., 2006; Helmstetter et al., 2008; Wanisch and Wotjak, 2008), and from these studies it is clear that the magnitude, diffusion and time course of protein synthesis inhibition varies with the route and dosage of drug administration as well as with targeted brain regions. Thus, to proceed with the rigorous assessment and interpretation of the PSI-induced behavioural effects in the present experiments, the magnitude, diffusion, and temporal decay of protein synthesis inhibition resulting from the intra-hippocampal infusions of anisomycin were first assessed by means of autoradiographic imaging and quantitative densitometric analysis of [^{14}C] L-leucine uptake. The analysis of incorporation of radiolabelled amino acids into protein has been a widely used tool in the field of learning and memory. It has been used for purposes such as detecting brain regional changes in protein synthesis after learning (Hyden and Lange, 1968; Beach et al., 1969; Hyden and Lange, 1969, 1970b, a; Yanagihara and Hyden, 1971; Hyden and Lange, 1972b, a; Pohle and Matthies, 1974; Hyden et al., 1977), assessing protein synthesis in rodent hippocampal slices (Phillips and Steward, 1988; Lipton and Raley-Susman, 1999), detecting changes in protein synthesis after induction of synaptic plasticity (e.g. Fazeli et al., 1993) and

more recently, vastly used for assessing protein synthesis inhibition in the brain following systemic or central administration of broad spectrum protein synthesis inhibitors in memory and synaptic plasticity studies (Flood et al., 1973; Davis et al., 1980; Patterson et al., 1989; Rosenblum et al., 1993; Meiri and Rosenblum, 1998; Maren et al., 2003; Ben Mamou et al., 2006; Milekic et al., 2006; Morris et al., 2006; Helmstetter et al., 2008; Wanisch and Wotjak, 2008; Abbas et al., 2009).

In this study, intra-hippocampal infusions of anisomycin were shown to produce a robust, long-lasting, inhibition of protein synthesis that was largely circumscribed to the target area, i.e. the dorsal pole of the hippocampus (see Fig. 3.16). The effect of anisomycin declined as a function of the time after infusion and the distance from the infusion site. Substantial levels of inhibition were still detected in this brain region 6-7h after infusion (~45% inhibition), but were fully reversed after 24h. Despite the highly localized effect of the drug, there was some evidence of partial inhibition in extra-hippocampal regions such as the thalamus and overlying neocortex; which has also been observed in previous studies (Morris et al., 2006). The implications of these findings to both Expt. 18 and 20 of this study are as follows: *First*, this establishes that encoding events given after anisomycin infusions occurred under strong translational arrest (>95%) in the dorsal hippocampus. The blockade of memory consolidation has been proposed to require levels of PS inhibition >90% (Davis and Squire, 1984). *Second*, retention of memory for those events was assessed under partial inhibition of protein synthesis in that brain region (~45%). *Third*, protein synthesis inhibition was fully reversed 24h later, so it could not have interfered with learning or memory consolidation during subsequent training days.

Intra-hippocampal infusions of the protein synthesis inhibitor anisomycin were shown not only to produce reversible translational arrest in the dorsal hippocampus but also to disrupt the formation of long-term memory in the DMP task in the watermaze. These results support a vast literature suggesting that protein synthesis is required for the formation of long-term memory

(Flexner et al., 1963; Agranoff et al., 1965; McGaugh, 1966; Flexner et al., 1967; Hyden and Lange, 1969; Davis and Squire, 1984; Goelet et al., 1986), namely for the formation of long-term place memory in the watermaze (e.g. Guzowski and McGaugh, 1997; Meiri and Rosenblum, 1998; Guzowski et al., 2000; Guzowski et al., 2001; Gusev et al., 2005; Plath et al., 2006; McGauran et al., 2008). In experiment 20, long-term memory for strong encoding events was disrupted when anisomycin was administrated 30min before, but not after, training. This suggests that proteins required for the consolidation of memory were synthesised at least within one hour after training. Expression of Arc protein in hippocampal neurons, which is up-regulated by strong encoding events (see Expt. 21) and can be blocked by anisomycin (Miyashita et al., 2008), is maximal within 30-90min after a single spatial exploration session (Ramirez-Amaya et al., 2005). The fact that Arc protein expression in the hippocampus is required for the consolidation of reference place memory in the watermaze (Guzowski et al., 2000) suggests that Arc protein may be a late-phase plasticity-related protein required for the consolidation of memory for strong encoding events in the present study.

Although anisomycin was shown to produce robust inhibition of protein synthesis in the hippocampus, the assumption that anisomycin-induced amnesia resulted from translational arrest in that brain region is open to two main lines of criticism. *First*, non-specific effects of anisomycin might have contributed to memory loss. *Second,* amnesia may have been a state dependent effect.

Non-specific effects of anisomycin

Broad-spectrum protein synthesis inhibitors (PSI) such as puromycin, ementine, cycloheximide, acetoxycycloheximide and anisomycin became standard tools in the study of learning and memory after seminal studies established a link between the central or systemic administration of these compounds and the selective disruption of long-term memory formation (reviewed by Davis and Squire, 1984). Since then a multiplicity of studies have built on the

premise that PSI-mediated effects are mediated by translational arrest to conclude in favour of a requirement for protein synthesis in consolidation and re-consolidation of memory (recently reviewed by Tronson and Taylor, 2007; Alberini, 2008; Hernandez and Abel, 2008; Nader and Hardt, 2009), namely allocentric place memory (e.g. Meiri and Rosenblum, 1998; Naghdi et al., 2003; Morris et al., 2006; Rossato et al., 2006; Flint et al., 2007; Artinian et al., 2008; Rodriguez-Ortiz et al., 2008). However, almost as soon as the first reports of PSI-induced amnesia were published concerns were raised regarding multiple side effects of the drugs and the validity of the conclusions derived from their use. Sickness, conditioned aversion, alterations in spontaneous locomotor activity, disruption of cerebral electrical activity and changes in catecholamine biosynthesis, were all side effects reported in early studies. Still, assurance in the use of PSIs, and specifically in the use of anisomycin, supposedly the less toxic of the compounds (Flood et al., 1973; Squire and Barondes, 1974; Flood et al., 1975; Stork and Welzl, 1999), was reinstated after different lines of evidence dissociated side effects from those ostensibly producing amnesia (reviewed by Davis and Squire, 1984; but see Gold, 2006). In the present study the fact that anisomycin produced no effects on swim speed or escape latencies during acquisition trials (Expt. 18 and 20, see Fig. 3.15b-c) suggests that motivational and sensorimotor processes underlying performance in the watermaze were intact. This was reported by others (Meiri and Rosenblum, 1998; Naghdi et al., 2003; Morris et al., 2006; Rodriguez-Ortiz et al., 2008), as well as the absence of effect of intra-hippocampal infusions of anisomycin (80 μg/μl *per side*) on activity/exploration in an open field and anxiety in an elevated plus maze (Vianna et al., 2003). Recently, however, new findings have added to the original scepticism and non-specific effects of anisomycin, such as apoptosis, have again been proposed to mediate anisomycin-induced amnesia rather than inhibition of protein synthesis *per se* (Rudy et al., 2006; Radulovic and Tronson, 2008; Qi and Gold, 2009).

Evidence from *in vitro* studies indeed suggest that anisomycin can induce apoptosis by activating c-Jun-terminal (JNK) and p38 stress-activated protein kinases, two sub-types of mitogen-activated protein (MAP) kinases that respond to inflammatory cytokines and cellular stress by promoting inflammation and cell death (Iordanov et al., 1997; Schaeffer and Weber, 1999; Shifrin and Anderson, 1999; Curtin and Cotter, 2002; Stadheim and Kucera, 2002; Eguchi et al., 2007). However, the exclusive role of JNK and p38 kinases in cell death remains controversial. A considerable number of reports have shown that depending on cell lines, culture conditions and states of cell differentiation, activation of these kinases might actually lead to cell differentiation and survival rather than cell death (reviewed by Nozaki et al., 2001; Hernandez and Abel, 2008). Furthermore, there is some evidence suggesting that anisomycin might also activate extracellular-signal regulated (ERK) kinases (Dhawan et al., 1999; Hong et al., 2007), a third sub-type of MAP kinases which promote cell development, growth and survival (Seger and Krebs, 1995; Hetman and Gozdz, 2004). A dynamic balance between activation of ERK and JNK/p38 has been shown to determine survival or apoptosis in some cell lines (Xia et al., 1995). Also, activation of both ERK and JNK has been proposed to mediate anisomycin-induced protective effects observed following a diversity of cellular insults (Dessi et al., 1992; Barancik et al., 1999; Hong et al., 2007). In view of this, the possibility that *i)* apoptosis occurs in the brain following administration of anisomycin *in vivo* and that *ii)* cell death contributes to amnesia (Rudy et al., 2006), remains speculative, while anisomycin-induced inhibition of protein synthesis is well documented (Flood et al., 1973; Davis et al., 1980; Patterson et al., 1989; Rosenblum et al., 1993; Meiri and Rosenblum, 1998; Maren et al., 2003; Ben Mamou et al., 2006; Morris et al., 2006; Helmstetter et al., 2008; Wanisch and Wotjak, 2008). Previously published studies found only little or no evidence of gliosis or cell loss after intra-ventricular, intra-hippocampal and intra-amygdalar infusions of anisomycin (Santini et al., 2004; Morris et al., 2006; Canal and Gold, 2007). Histological analysis of brain sections in this study supports these findings; no significant cell loss was observed in the hippocampus beyond the physical damage of cannulae placement (Expt. 18-20; see Fig. 3.15e).

That cell death is not a major contributor to anisomycin-induced amnesia is further suggested by two lines of evidence. *First*, both *in vitro* and *in vivo* studies have shown that anisomycin can act as a neuroprotective agent (Shigeno et al., 1990; Finnegan and Karler, 1992) and even prevent neuronal apoptosis when administered systemically at doses known to produce amnesia (Lopez-Mascaraque and Price, 1997). *Second*, there is a vast literature showing that *i)* anisomycin does not produce amnesia when administered within a restricted time window after training and that *ii)* anisomycin infusions do not prevent new learning (Squire and Barondes, 1974; Meiri and Rosenblum, 1998; Duvarci and Nader, 2004; Morris et al., 2006; Parsons et al., 2006; Rossato et al., 2006). The results obtained in this study also support some of these findings. *First*, in experiment 20, anisomycin was shown to disrupt formation of long-term memory when administered 30min before, but not after, encoding. *Second*, both in experiments 18 and 20, infusions of anisomycin did not impair learning during subsequent training days, when protein synthesis was restored (Expt.19). *Third*, levels of performance were stable throughout the duration of experiments comprising one (Expt. 18) or more (Expt. 20) infusions of anisomycin. If amnesic effects were due to cell toxicity and subsequent cell death then anisomycin would have been expected to *i)* disrupt memory at any time point after encoding, *ii)* prevent subsequent learning and *iii)* produce cumulative impairment of performance over time. Rudy (Rudy, 2008) has recently suggested that temporally graded anisomycin-induced amnesia can be explained if *i)* drug infusions only kill a small number of local neurons (which are insufficient to produce detectable amnesia) and if *ii)* the locally-activated apoptotic cascade induces *"a state of hyper-excitability that disturbs the normal activity of the remaining neurons in the local neural circuitry needed to stabilize the memory trace"*. According to the author, depending on the temporal relationship to training cell death and hyper-excitability would be able to disrupt a fragile memory trace (during and immediately after training) but not a consolidated trace (later after training); connections between surviving neurons would support retrieval in the later case. An alternative *system level* explanation for graded amnesia would be that disturbed neurons would "*project an abnormal input to the region where the memory is*

stored" (Rudy, 2008). These hypotheses are based on assumptions that still require experimental validation and are difficult to reconcile with experiments showing two distinct, non-consecutive, time windows for the amnesic effects of both systemic and intra-hippocampal infusions of anisomycin (Grecksch and Matthies, 1980; Bourtchouladze et al., 1998; Quevedo et al., 1999). It is hard to conceive how cell death and hyper-excitability would disrupt a memory trace during or immediately after training, fail to disrupt it after an initial consolidation phase, and then disrupt it again at a later stage of consolidation.

Finally, anisomycin has been proposed to produce amnesia by producing abnormal release of neurotransmitters (Gold, 2006; Qi and Gold, 2009) and by leading to abnormal induction of mRNA (Radulovic and Tronson, 2008). These may be potential shortcomings of the present work (but see Davis and Squire, 1984; Alberini, 2008; Hernandez and Abel, 2008).

State dependency

State dependency (Overton, 1964; Izquierdo, 1984) is unlikely to account for the amnesic effects of anisomycin. *First*, infusions of anisomycin before encoding prevented retrieval of memory when the drug was still active (Expt. 18-20; see Fig. 3.16). *Second*, in experiment 20, the same infusion of anisomycin was shown to disrupt retrieval of memory for subsequent, but not preceding encoding events, when protein synthesis inhibition could still be observed in the hippocampus. These results are consistent with previous studies suggesting that intra-cerebral infusions of anisomycin do not induce state-dependent learning (Patterson et al., 1989; Santini et al., 2004).

4.7. Contribution of synaptic tagging and capture mechanisms to the formation of place memory

The main objective of this thesis was to investigate whether, as predicted by the STC hypothesis, STC mechanisms could be detected during the formation of long-term memory, and specifically, during the formation of long-term allocentric place memory. First, encoding of rapidly acquired allocentric place memory was shown to depend on the activation of NMDA receptors in the dorsal hippocampus. Next, consolidation of memory was shown to depend on translational mechanisms in that same brain region. Strong encoding events in the watermaze, leading to the formation of long-lasting protein synthesis-dependent allocentric place memory, were shown to induce both transcriptional and translational mechanisms in the dorsal hippocampus. Finally, new behavioural protocols in the watermaze, combining different strong encoding events, similar to "strong-before-strong" STC electrophysiological paradigms (Frey and Morris, 1997), or strong and weak encoding events, similar to "strong-before-weak" or "weak-before-strong" paradigms (Frey and Morris, 1997, 1998b), failed to reveal synergistic interactions between events. Possible explanations for the failure to find behavioural correlates of STC mechanisms in the watermaze are as follows:

General explanations

1) Synaptic tagging and capture mechanisms may not occur *in vivo*;
2) The properties of synaptic tagging and capture may not extend to behavioural memory;
3) The putative contribution of STC mechanisms to the formation of memory (Moncada and Viola, 2007) may not generalise to all types of memory.

Explanations specific to protocols in the watermaze

4) High levels of arousal or novelty may be required to trigger synergistic interactions, which were not induced by the strong modulatory events used in the present experiments;
5) Overlap of neuronal ensembles recruited by the different events might have been insufficient to produce detectable synergistic interactions.

Explanations specific to "strong-before-weak" and "weak-before-strong" protocols

6) Weak encoding events may not set tags;
7) In the "weak-before-strong" paradigm the interval between events may have been too long.

General explanations

Although the study by Moncada and Viola (2007) is strongly suggestive that STC-like mechanisms may contribute to the formation of memory and, therefore, that STC mechanisms may occur *in vivo*, no conclusive evidence has been provided by this, or any other study, that such is the case. In view of this, the present results may reflect the absence of STC mechanisms *in vivo*. Alternatively, STC mechanisms may occur *in vivo* and contribute to the formation of memory traces but its principle, or properties, possibly as other general properties of synaptic plasticity (Martin et al., 2000b; Bliss et al., 2007), may not have a direct translation into observable behaviour. Manifestations of memory are not only due to synaptic properties; they also depend on the network in which the plasticity is embedded. As reviewed in the Introduction, several studies have tried to expose STC-like mechanisms during the formation of different types of memory supported by different brain structures. STC-like mechanisms were only associated to hippocampus-dependent inhibitory avoidance learning (Moncada and Viola, 2007). It is possible that the contribution of STC mechanisms to the formation of memory does not generalise to all types of memory, including different types of hippocampal-dependent memories.

Specific to the newly developed protocols in the watermaze

While describing the formation of retroactive hypermnesia Stratton (1919) referred that "vivid recollections" would be mostly determined by the intensity rather than the quality of the "emotional excitement". For Brown and Kulik (1977) the creation of flashbulb memories depended on high levels of "surprise" and "arousal". It is indeed possible that high levels of arousal or novelty are required to trigger STC-like synergistic interactions and that these were not induced by the strong "modulatory" events used in the present experiments in the watermaze. In the Moncada and Viola (2007) study the memory rescuing effect was dependent on the novelty of the environment and mediated by dopaminergic neuromodulation in the hippocampus, which is thought to be involved in novelty processing (Lisman and Grace, 2005). In the DMP task in the watermaze, the position of the platform is also novel every day and formation of long-term memory for its position is dependent on the activation of dopamine receptors in the hippocampus (O'Carroll et al., 2006). Still, after pretraining, neither the environment, nor the task, nor the "new" platform positions, are "too novel" or produce high levels of arousal. The strong encoding events in the watermaze were shown to induce both transcriptional and translational mechanisms in the hippocampus, and consisted of spaced training to potentiate those mechanisms (e.g. Kogan et al., 1997; Josselyn et al., 2001; Genoux et al., 2002; Scharf et al., 2002). However, up-regulation of protein synthesis was relatively weak. For example, up-regulation of Homer 1a mRNA was observed in 11-14% of CA1 hippocampal cells. By comparison, a 6min spatial exploration of a novel environment, similar to that used by Moncada and Viola (2007), up-regulates Homer 1a mRNA in ~30-35% of those cells (Vazdarjanova and Guzowski, 2004). High arousal, or novelty, may recruit additional cellular mechanisms that result in the higher expression of protein synthesis, thus facilitating STC mechanisms and their behavioural expression. Related to this possibility is also the alternative explanation that the levels of overlap obtained between the neuronal ensembles recruited by two different encoding events, occurring in different watermaze rooms, were too

low to produce detectable synergistic interactions. In addition to the possibility that protein synthesis was not up-regulated in a sufficient number of cells, remapping between environments may have also contributed to the recruitment of less-overlapping neuronal ensembles (Colgin et al., 2008). This is a considerable problem when investigating behavioural correlates of STC mechanisms, because in the absence of detectable synergistic interactions the argument can always be made that overlap was not "strong enough" even when some degree of overlap is shown.

Specific to "strong-before-weak" and "weak-before-strong" protocols

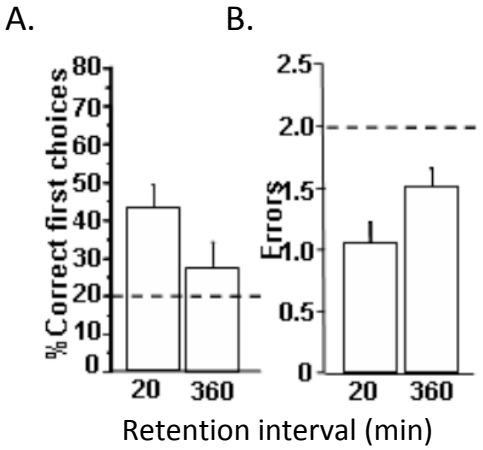
In the seminal study describing synaptic tagging and capture mechanisms in the CA1 region of rat hippocampal slices, Frey and Morris (1997) have shown that the strength of the weak tetanic stimulation protocols inducing E-LTP was critical for the conversion of E-LTP to L-LTP. If the weak stimulation was not "strong" enough E-LTP would not be stabilized, suggesting that no tags had been produced. It is possible that weak encoding events in the present protocols produce short-term memory but do not set tags, this would explain the absence of memory rescue. A further explanation for the failure to detect a synergistic interaction between encoding events in the "weak-before-strong" paradigm may be that the interval between the events was too long. Although the tag is thought to have a lifetime of about 1-2h in rat hippocampal slices maintained at 32^0C (Frey and Morris, 1998b), which falls within the time interval anticipated in the present experiments (i.e. with events separated 1h apart), studies of structural and behavioural reinforcement carried in Frey's lab suggest that the lifetime of the tag may only be 30min in the intact animal (reviewed in Reymann and Frey, 2007). Although this is a possibility, it is hard to reconcile with Moncada and Viola's study (2007) in which memory for inhibitory avoidance was stabilized by the exploration of a novel environment 1h earlier in a protein synthesis-dependent manner. Neither of these two possibilities can explain the absence of detectable synergistic interactions in the "strong-before-strong" paradigm.

Supplemental data

Supplement 1. *Expt. 3: First choices and errors.*

When averaged over 6 trials (4 standard and 2 probe trials), the percentage of first choices significantly differed from chance at 20min [$t_{(15)}$=3.80, P<0.005] but not at 360min [$t_{(15)}$=1.27, P=0.22] (see below). The average number of errors significantly differed from chance at both retention intervals [t(15)>3.16, P<0.01]. Still, no significant effect of retention interval was observed with first choices or errors [$t_{(15)}$<1.7, P>0.11]. If probe trials were analyzed separately, the difference between retention intervals closely approached significance for the first choice measure [20min: 34.38+5.98, 360min: 15.63+5.98; $t_{(15)}$=2.09, P=0.054] and reached significance for the error measure [20min: 0.63+0.16, 360min: 1.96+0.22; $t_{(15)}$=2.47, P<0.05]; moreover, in probe trials, both first choice and error measure significantly differed from chance at 20min [$t_{(15)}$>2.40, P<0.05], but not at 360min [$t_{(15)}$<1]. While the difference between probe and standard trials in this experiment may reflect that rats' performance was supported by odour cues emanating from the food reward in standard trials, it is more likely to reflect the relatively high between-trial variance of the first-choice and error measures.

Expt. 3: Retrieval of one-trial place memory after arena rotation. In order to establish that performance did not rely on cryptic odor cues, one-trial place memory was assessed after the arena was rotated between encoding and retrieval phases. Retention of memory was investigated 20min and 360min after acquisition. The first-choice (***A***) and error (***B***) measures of performance are shown. Data is collapsed over 6 trials (4 standard trials and 2 probe trials). Stippled horizontal lines indicate chance values of performance measures. Mean±1SEM.

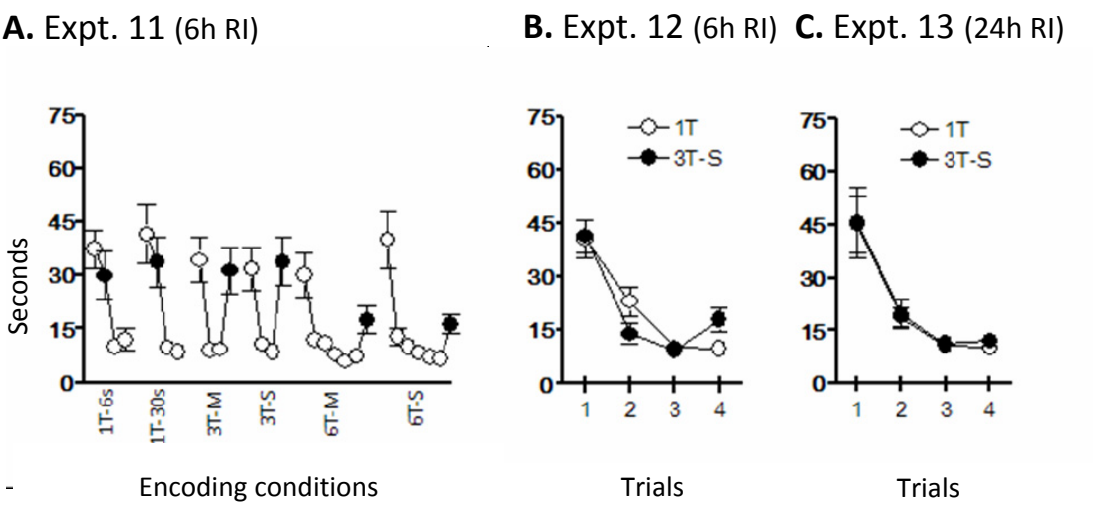


Supplement 2. *Expt. 6: Pre-testing and mock infusions*

A) The percentage of correct first choices and the number of errors obtained in the 3 trials (2 standard training trials plus 1 probe trial) were significantly different from chance both at 5 and 45 minutes [first choices: $t_{(14)}>2.9$, $p<0.004$; errors: $t_{(14)}>5.6$, $p<0.0001$], with no difference between retention intervals [first choices: $t_{(14)}<0.5$, $p>0.6$; errors: $t_{(14)}<1.0$, $p>0.3$]. In the probe trials, the percentage of dig time at the sandwells in correct locations was higher than the average percentage of dig time at sandwells in novel locations [$F_{(1,14)}=60.2$, $p<0.0001$], regardless of retention interval [no main effect or interaction: $F_{(1,14)}<1.2$, $p>0.29$]. The percentage of dig time spent at the correct sandwell was significantly higher than chance with both retention intervals and the average percentage of dig time spent at the sandwells in the novel locations was lower than chance [$t_{(14)}>3.9$, $p<0.002$].

B) After recovering from surgery, rats showed good performance in four habituation trials, during which they were familiarized with a 20min retention interval, as well as with the handling required for the infusion experiments. The average number of errors in the two trials with mock infusions (1.18±0.21) and the two trials without mock infusions (1.00±0.16) did not differ ($t_{(13)}=0.6$; $p>0.56$) and was significantly lower than the chance level ($t_{(13)}>3.9$; $p<0.002$).

Supplement 3. *Expt. 11-13: Probe day latencies*



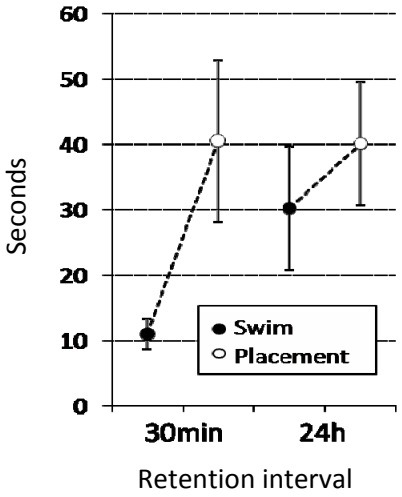
Expt. 11-13: Enhancement of long-term place memory strength by repetition and spacing of acquisition trials. Probe day latencies are shown. ***A)*** Expt. 11 assessed long-term memory [6h retention interval (RI)] strength for the following types of encoding event: 1 encoding trial with either 6s (1T-6s) or 30s (1T-30s) on platform; 3 encoding trials with 15s (massed; 3T-M) or 10min (spaced; 3T-S) inter-trial intervals; and finally, 6 encoding trials with 15s (massed; 6T-M) or 10min (spaced; 6T-S) inter-trial intervals. Filled black circles represent crossing latencies during probe trials ***B-C)*** Memory strength for a single encoding trial or 3 spaced encoding trials (10min ITI; 3T-S) was investigated in a new batch of animals after retention intervals of 6h (Expt. 12) or 24h (Expt. 13). Mean±1SEM.

Experiment 11: The fact that start positions were adjusted in each probe day to match retention trials and their preceding trial throughout conditions allows the analysis of crossing latencies but not of latency savings (*see Materials and methods*). Analysis of crossing latencies in probe days revealed a significant difference between conditions ($F_{(5.85)}$=2.4; p<0.05) (see above). Since *post-hoc* Fisher's PLSD tests did not show significant differences between conditions with the same number of encoding trials (p=0.62) these were grouped to reveal a main effect of trial number ($F_{(2.34)}$=6.39; p<0.005) and a highly significant difference between 6 encoding trials and either 1 or 3 encoding trials (p<0.005). Groups with 1 and 3 encoding trials did not differ (p=0.88). The average retention trial latency for the conditions comprising 1, 3 and 6 encoding trials was 31.9±5s, 32.7±5s and 16.9±2s, respectively. Performance in the condition where rats were given one trial and allowed to stay on the platform for 30s only

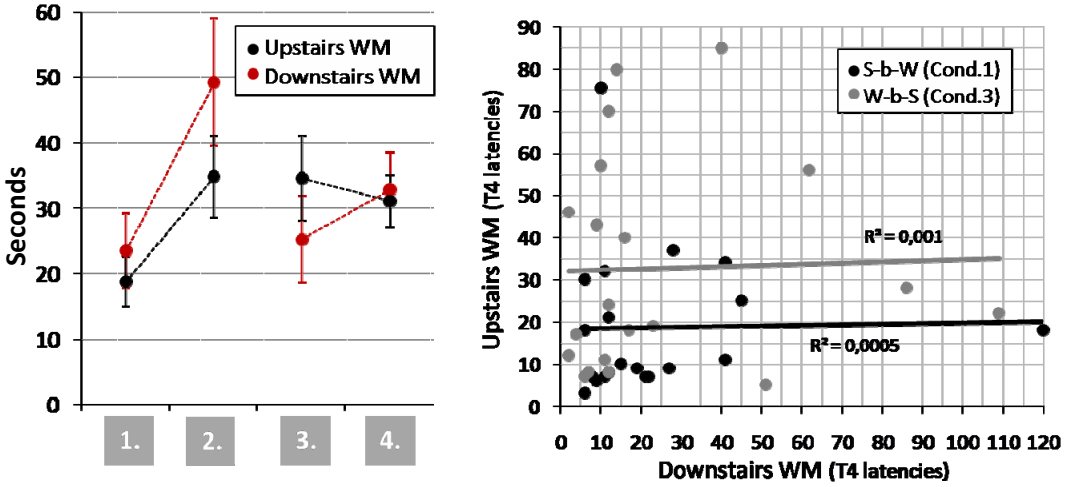
differed from performance obtained with either of the 6 trial conditions ($p<0.05$). Thus, analysis of the retention trial latencies only revealed an enhancing effect of the number of encoding trials on 6h memory.

Supplement 4. *Expt. 14: Crossing latencies*

Expt. 14: Crossing latencies. Rats were given 3 "swim" trials or 3 placement trials. Encoding trials were separated by 10min (spaced training). During a "swim" trial rats were allowed 2min to swim and find the platform and 30s on the platform. During a placement trial rats were only placed on the platform for 30s. Retention of memory for both "swim" and placement trials was tested 30min or 24h after acquisition. Mean±1SEM.

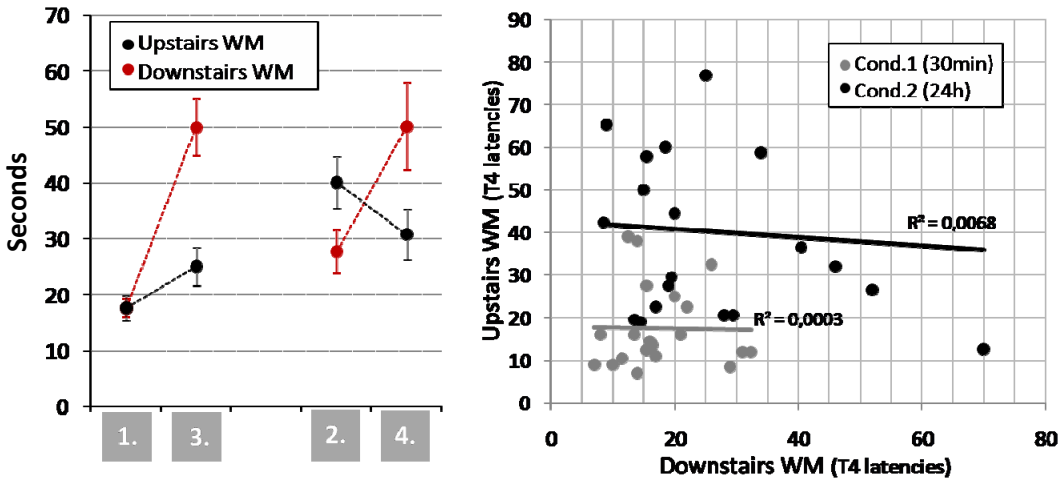


Supplement 5. *Expt. 15: Crossing latencies*



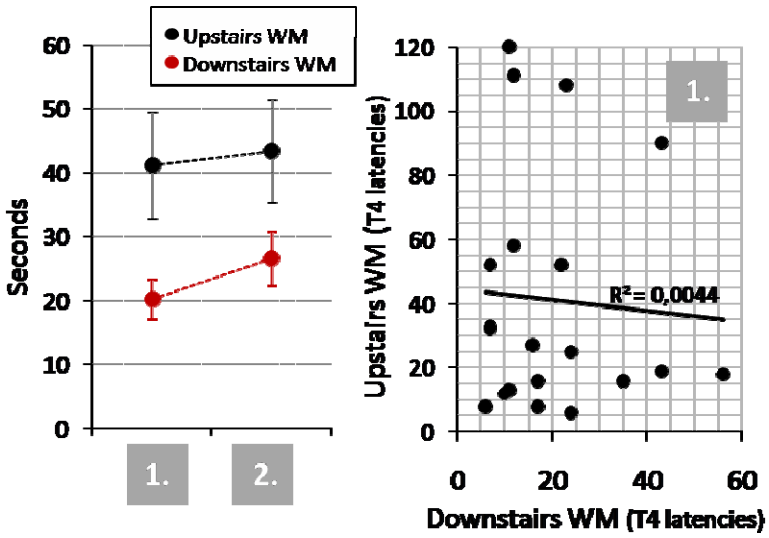
Expt. 15: Investigating synergistic interactions between strong and weak encoding events. Rats were given a weak encoding event in the upstairs watermaze (black) preceded (Cond. 1), or followed (Cond. 3), 50min apart, by a strong encoding event in the downstairs watermaze (red). Control conditions (2 and 4) replaced the strong encoding events by weak encoding events in the downstairs watermaze. *Left:* Crossing latencies are shown. Mean±1SEM. *Right*: Correlation of crossing latency values obtained in both watermazes for the strong-before-weak condition (black) and the weak-before-strong (gray) condition (Cond. 1 and 3).

Supplement 6. *Expt. 16: Crossing latencies*



Expt. 16: Investigating synergistic interactions between strong and weak encoding events. Rats were given a weak encoding event in the upstairs watermaze (black) preceded, 50min apart, by a strong (Cond. 1-2) or weak (Cond. 3-4) encoding event in the downstairs watermaze (red). Memory for the weak encoding events occurring upstairs was assessed 30min (Cond. 1 and 3) or 24h (Cond. 2 and 4) after acquisition. Memory for encoding events occurring downstairs (red) was assessed 26h after acquisition. *Left*: Crossing latencies during probe trials. Mean±1SEM. *Right*: Correlation of crossing latency values obtained in both watermazes in conditions 1 and 2. R^2 (R-squared).

Supplement 7. *Expt. 17: Crossing latencies*



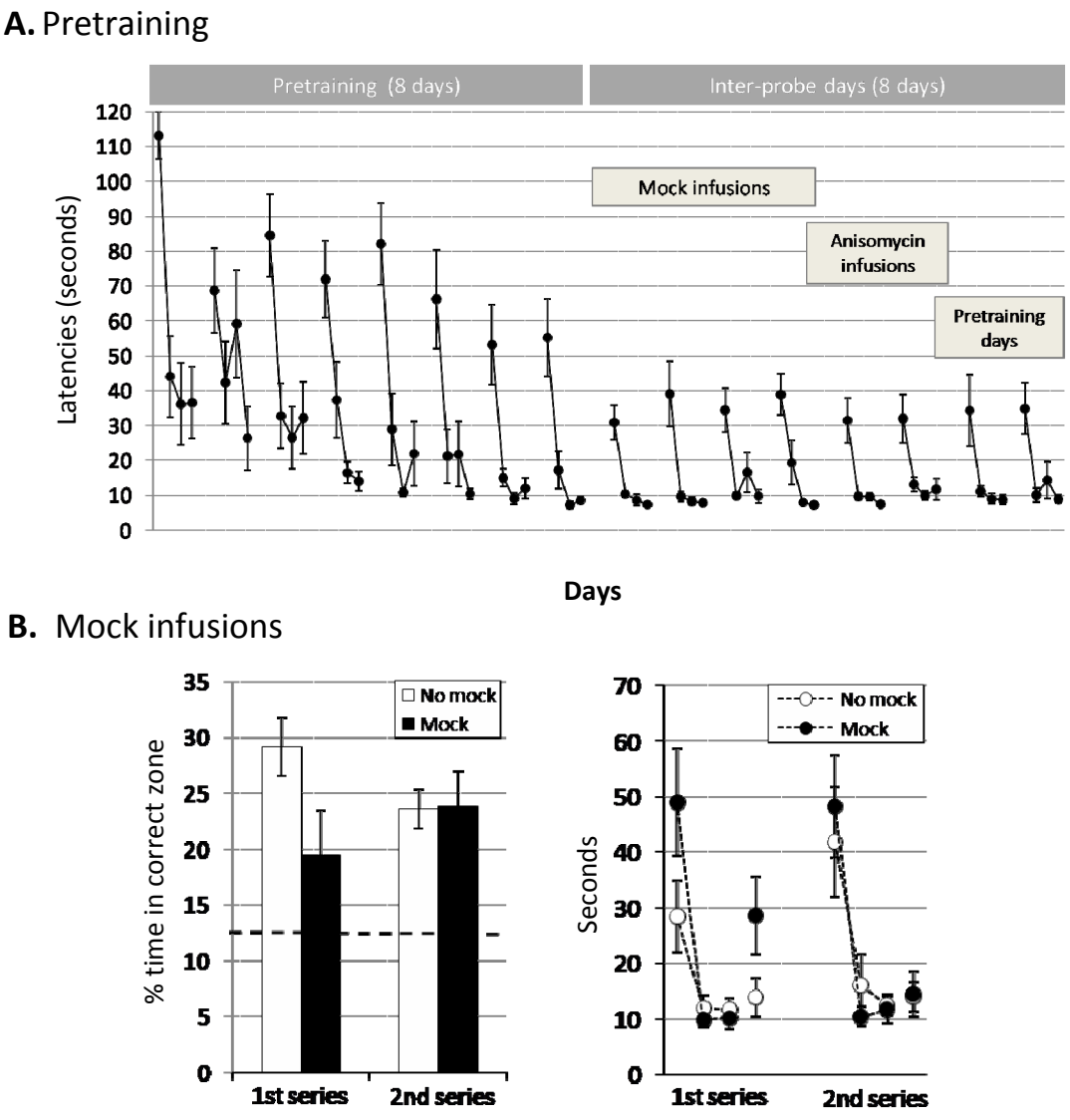
Expt. 17: Investigating synergistic interactions between strong and weak encoding events. Rats were given a weak encoding event in the upstairs watermaze (black) preceded, 5min apart, by a strong (Cond. 1) or weak (Cond. 2) encoding event in the downstairs watermaze (red). Memory for the weak encoding events occurring in the upstairs watermaze was assessed 24h after acquisition. Memory for the encoding events occurring in the downstairs watermaze was assessed 26h after acquisition. *Left:* Crossing latencies during probe trials. *Right*: Correlation of crossing latency values obtained in both watermazes in condition 1. R^2 (R-squared).

Supplement 8. *Expt. 18: Pretraining and mock infusions*

Pretraining: Task acquisition proceeded as described in previous experiments (e.g. Expt. 8-10; Fig. 3.7) Significant T1-T2 latency savings were observed from the first day of pretraining and T3-T4 escape latencies reached asymptotic levels of about 15s around days 4-6 (see A below). Asymptotic levels of performance were observed throughout inter-probe days.

Mock infusions: After pretraining, rats were given a first series of probe days which included mock infusions in order to assess and minimize any effect of infusion procedures on performance before relevant testing days. The two conditions tested in these probe days partially mimicked the main conditions of the experiment in which either aCSF or anisomycin (a protein synthesis inhibitor) were infused bilaterally in the dorsal hippocampi 30min before strong encoding events (3 spaced swim trials; 5min ITI); 6h retention interval; Fig. 3.15a. Instead of aCSF or anisomycin infusions animals were given either mock infusions, in which syringes and tubing were empty but were otherwise identical to "regular" infusions (see Material and methods), or no infusions (control condition). The analysis of the time that rats spent searching the correct zone during probe trials revealed an effect of mock infusions on long-term memory strength (6h) for strong encoding events in the first series of probe days ($t_{(11)}$=2.8; p<0.05; see B1 below). Above chance performance during probe trials was only observed when animals were not given mock infusions ($t_{(11)}$=6.9, p<0.0001; $t_{(11)}$=1.7, p=1.17 for mock infusions). Although rats given mock infusions took longer to reach/cross the platform on trials 1 and 4 (see see B2 below), the analysis of escape latencies during encoding trials ($F_{(1,11)}$=2.0, p=0.19) and of crossing latencies during the probe trial ($t_{(11)}$=2.0; p=0.07) failed to show a significant difference between drug and control conditions. Given that the first series of probe days revealed that animals required further habituation to infusion procedures in order to prevent interference with memory formation, a second series of probe days was carried out which repeated the exact same protocol. The second series of mock infusions did not produce detectable effects on memory

strength. Neither analysis of crossing latencies ($t_{(11)}$<1), nor zone analysis ($t_{(11)}$<1), revealed significant differences in performance between conditions (see B1-2 below). Above chance performance was detected for both conditions during probe trials ($t_{(11)}$=3.6, p<0.005) and no significant differences were observed in escape latencies during encoding trials ($F_{(1,11)}$<1).



Expt. 18: Task acquisition and mock infusions. ***A)*** Rats were given 8 pretraining days (4 trials/day; 15s ITI). ***B)*** Transitory effect of mock infusions on performance. *Left:* Percentage of time rats spent searching the correct zone during probe trials. Stippled horizontal lines indicate chance level. *Right:* Latencies obtained in the first and second series of probe days testing for the effect of mock infusions on 6h memory for 3 spaced encoding trials (5min ITI). Mean±1SEM.

Supplement 9. *Expt. 19: Tracer concentrations*

Uptake of [^{14}C] L-leucine into hippocampus following local infusion of anisomycin or aCSF

	Tracer concentration (nCi/g)				
Anterior sections	CA1	CA2	CA3	DG	Total HPC
30min	*	*	*	*	*
Ipsilateral (aCSF)	3 ± 3	2 ± 1	3 ± 2	7 ± 6	2 ± 2
Contralateral (anisomycin)	130 ± 6	153 ± 7	150 ± 6	176 ± 8	115 ± 10
3h45min	*	*	*	*	*
Ipsilateral (aCSF)	14 ± 10	10 ± 8	10 ± 6	28 ± 20	22 ± 10
Contralateral (anisomycin)	125 ± 7	158 ± 6	144 ± 7	184 ± 9	121 ± 15
6h45min	*	*	*		*
Ipsilateral (aCSF)	45 ± 7	47 ± 15	87 ± 16	121 ± 28	62 ± 11
Contralateral (anisomycin)	130 ± 5	152 ± 4	150 ± 4	177 ± 6	111 ± 12
24h45min					
Ipsilateral (aCSF)	120 ± 10	145 ± 12	139 ± 10	171 ± 11	116 ± 14
Contralateral (anisomycin)	127 ± 8	149 ± 10	140 ± 7	171 ± 10	118 ± 10
Posterior sections	CA1	CA2	CA3	DG	Total HPC
30min			*	*	
Ipsilateral (aCSF)	77 ± 22	112 ± 15	87 ± 24	51 ± 21	68 ± 25
Contralateral (anisomycin)	125 ± 5	142 ± 8	155 ± 10	160 ± 8	111 ± 11
3h45min	*			*	
Ipsilateral (aCSF)	72 ± 15	120 ± 20	110 ± 18	42 ± 15	90 ± 17
Contralateral (anisomycin)	130 ± 6	144 ± 5	150 ± 6	155 ± 8	126 ± 9
6h45min	*				
Ipsilateral (aCSF)	110 ± 5	138 ± 9	138 ± 13	142 ± 5	115 ± 8
Contralateral (anisomycin)	128 ± 5	140 ± 6	151 ± 8	157 ± 6	122 ± 10
24h45min					
Ipsilateral (aCSF)	107 ± 4	138 ± 3	146 ± 4	156 ± 3	119 ± 8
Contralateral (anisomycin)	111 ± 5	135 ± 2	141 ± 2	153 ± 4	117 ± 7

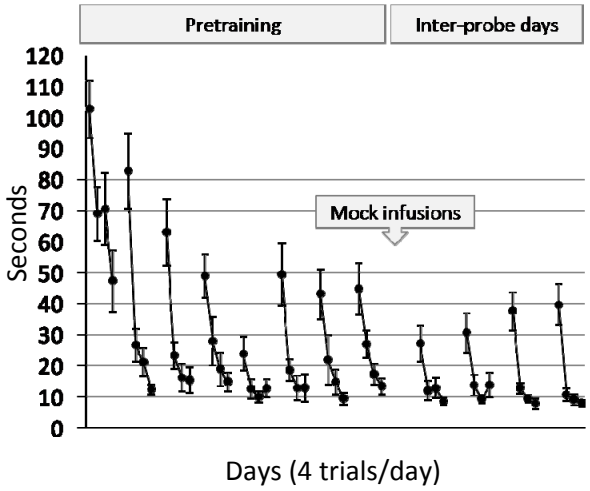
Data are presented as Mean±1SEM (*p<0.05; paired t-test).

Supplement 10. *Expt. 20: Pretraining, mock infusions and crossing latencies during probe trials*

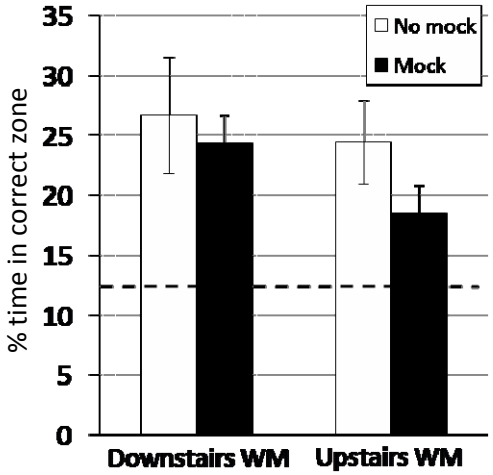
Pretraining (days 1-8): Task acquisition proceeded as previously described for Expt. 18 (see *Supplement 9a*), namely, T1-T2 latency savings were observed from the first day of pretraining and T3-T4 escape latencies reached asymptotic levels of about 15s around days 4-6 (see A below). Asymptotic levels of performance were also observed throughout inter-probe days (days 11, 13, 15 and 17, see A below).

Mock infusions (days 9-10): After pretraining, rats were given two probe days to assess potential effects of infusion procedures on performance. The conditions tested in these probe days mimicked the conditions of the experiment in which either aCSF or anisomycin infusions were administrated between two strong encoding events occurring in different watermazes (Cond. 3-4; see Fig. 3.17a). Instead of aCSF or anisomycin infusions animals were given either mock infusions (see Materials and methods), in which syringes and tubing were empty but were otherwise identical to "regular" infusions, or no infusions. Neither zone analysis ($F_{(1.15)}$=3.2; p=0.092; see B below), nor analysis of crossing latencies ($F_{(1.15)}$<1; *data not shown*) revealed a main effect of mock infusions on performance.

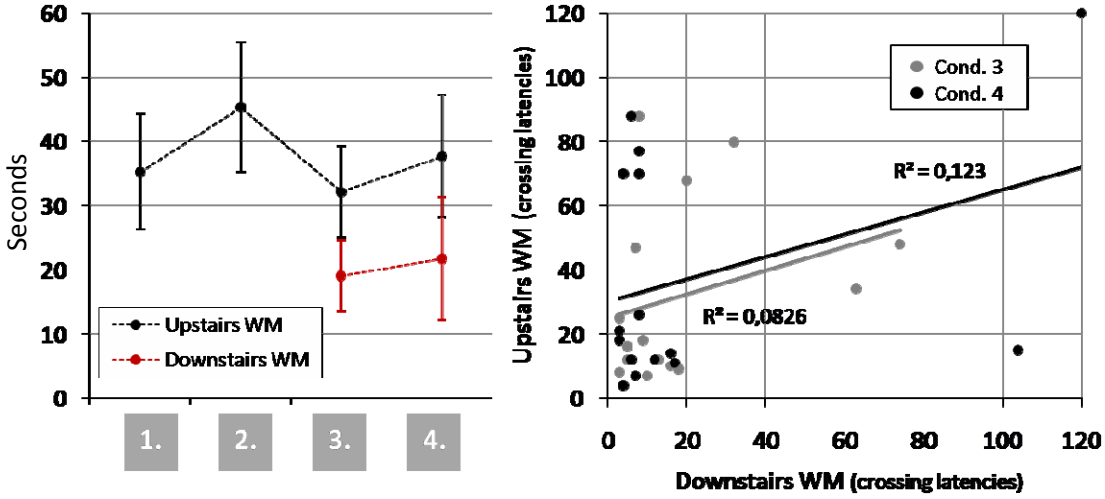
A. Pretraining (escape latencies)



B. Mock infusions



C. Probe trials (crossing latencies)



Expt 20: Investigating a behavioural analogue of the strong-before-strong paradigm in the watermaze. In conditions 1 and 2 rats were given a strong encoding event in the upstairs watermaze (black) preceded (30min apart) by bilateral intra-hippocampal infusions of aCSF (Cond. 1) or anisomycin (Cond. 2; 125µg/µl; 1µl per side). In conditions 3 and 4 rats were given two strong encoding events, one hour apart, in different watermazes located in different rooms. Bilateral intra-hippocampal infusions of aCSF (Cond. 3) or anisomycin (Cond. 4) occurred 30min after the first encoding event (downstairs watermaze; red) and before the second encoding event (upstairs watermaze; black). Memory for the strong encoding events occurring in the upstairs watermaze was assessed 6h after acquisition. Memory for the strong encoding events occurring in the downstairs watermaze was assessed 8h after acquisition. Left: Crossing latencies obtained during probe trials. Mean±1SEM. *Right*: Correlation of crossing latency values obtained in both watermazes in conditions 3-4. R^2 – R-squared.

Supplement 11. *Expt. 21: Behavioural data*

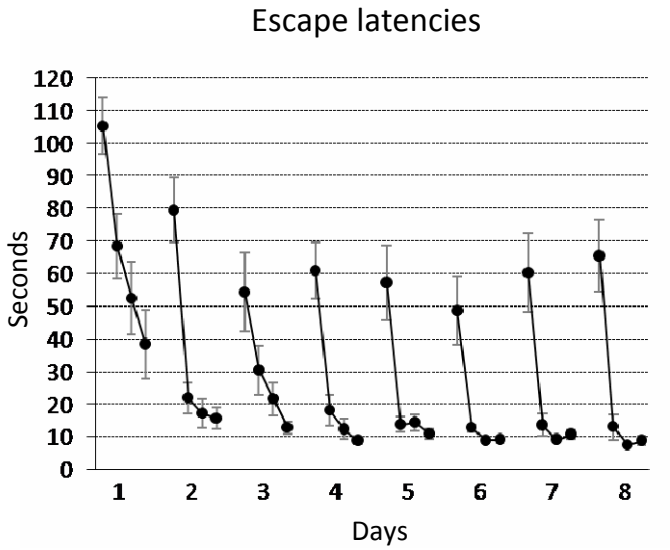
Pretraining (Cond. 2-5; days 1-8): Rats were first given 8 days of pretraining alternating between watermazes. The rate of task acquisition was comparable to that observed in Expt. 20 (see *Supplement 11a*); one-trial learning was observed from the first day of pretraining and T3-T4 escape latencies reached asymptotic levels of about 10-15s around days 4-6 (see A below).

Probe trials (Cond. 2-5; days 9-10): After pretraining, rats were given two probe days to: *i)* match the extent of training given to animals used in this experiment and Expt. 20 before testing days (i.e. day 11 and onwards); *ii)* compare the levels of performance between experiments obtained at that point of training; *iii)* and compare levels of performance between watermazes (upstairs and downstairs). Each day rats were given two "strong" encoding events (3 swim trials; 5min ITIs) occurring in different watermaze rooms 60min apart. Memory for the first encoding event was assessed 8h after encoding and memory for the second encoding event was assessed 6h after encoding. The design was counterbalanced so that performance for both encoding events could be compared between days and watermazes. Although zone analysis revealed a significant decline of performance between days for both encoding events ($F_{(1.15)}$=6.1; p<0.05), the time that rats spent searching the correct zone during probe trials assessing memory for the first and second encoding events was of 36.5±14.4% and 31.2±12.8% on day 1, and 30.0±9.5% and of 25.6±10.6% on day 2, respectively (see B below). When averaged between days, these levels of performance did not differ from those observed in probe trials assessing memory for conditions without mock infusions in Expt. 20 [see *Supplement 11b white bars*; planned *unpaired* t-tests: event 1 ($t_{(29)}$=1.3; p=0.2); event 2 ($t_{(29)}$<1)]. Interestingly, zone analysis revealed only a trend for a main effect of event on performance ($F_{(1.15)}$=3.2; p=0.09; see B below). As in this experiment both encoding events occurred in each of the two watermazes in a counterbalanced manner and in Expt. 20 "modulatory" events occurred always

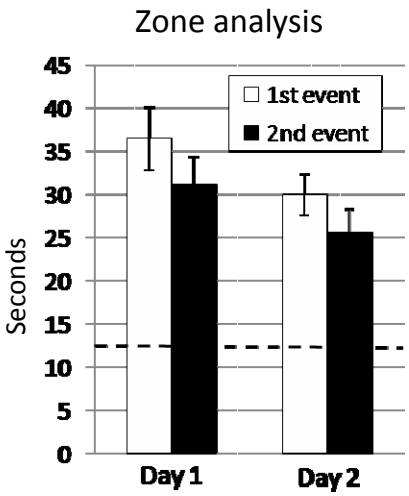
in the downstairs watermaze and "target" events occurred always in the upstairs watermaze, this suggests that the higher levels of performance observed for the "modulatory" events in Expt. 20 might have been, at least partially, due to a general difference in performance between watermazes. In the present experiment, however, the between-subjects comparison of the percentage of time rats spent searching the correct zone during probe trials, did not reveal a significant difference in performance between watermazes in either of the encoding events, in any of the probe days [planned *t*-tests: day 1, event 1 ($t_{(7)}$<1); day 1, event 2 ($t_{(7)}$=1.8; p=0.11); day 2, event 1 ($t_{(7)}$=2.2; p=0.07); day 2, event 2 ($t_{(7)}$=2.2; p=0.07)]. Analysis of crossing latencies did not reveal a main effect of day, or event on performance, as well as no difference in performance between watermazes (*data not shown*).

Testing day (Cond. 3 and 5; day 11): Before sacrifice, rats tested in Cond. 3 and 5 were given two "strong" encoding events (3 swim trials; 5min ITIs) 10min apart. In Cond. 3 both encoding events occurred in a single watermaze room; in Cond. 5, each encoding event occurred in a different watermaze room. Platform positions were always changed between encoding events in both conditions. Although a formal statistical analysis of performance measures is not presented, due to the reduced sample size (n=4 *per* condition), it is worth noting that escape latencies seem to reflect poor one-trial learning for the second platform position in Cond. 3 (see C above). This temporary impairment in performance, which seems to disappear by the third encoding trial, is most likely an effect of proactive interference and stresses the relevance of using two different watermaze rooms in the above described experiments investigating behavioural analogues of "synaptic tagging" paradigms.

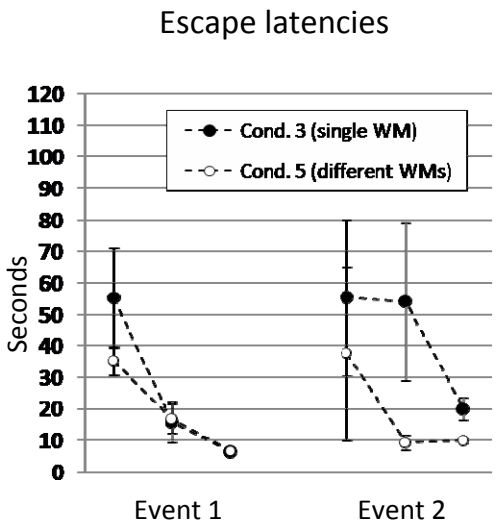
A. Pretraining (days 1-8)



B. Probe days (days 9-10)



C. Testing day (day 11)



Expt. 21: Behavioural data. ***A)*** Pretraining (Cond. 2-5): Rats (n=16) were given 8 days of pretraining, alternating between watermazes (4 trials/day; 15s ITIs). ***B)*** Probe days (Cond. 2-5): Each day rats were given two "strong" encoding events (3 swim trials; 5min ITIs), in different watermaze rooms. Encoding events were separated by 60min. Memory for the first encoding event (*white bars*) was assessed 8h after encoding; memory for the second encoding event (*black bars*) was assessed 6h after encoding. ***C)*** Testing day (Cond. 3/5): Rats were given two "strong" encoding events (3 swim trials; 5min ITIs), 10min apart, before sacrifice (*n*=4 *per* condition). In Cond. 3 (*black circles*) both events occurred in the same watermaze room; in Cond. 5 (*white circles*), the events occurred in different watermaze rooms. In both conditions the platform position was moved between encoding events. Mean ±1SEM.

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